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(54) Title: NUCLEIDE LABELLED H-TETRAZINES AND USE THEREOF FOR PET AND SPECT PRETARGETED IMAGING AND RADIONUCLEIDE THERAPY

(57) Abstract: The present invention relates to novel tetrazine compounds for use in pretargeted in vivo imaging and in therapy and to the precursors of the tetrazine compounds. The compounds are suitable for use in click chemistry, i.e. reactions that join a targeting molecule and a reporter molecule. The compounds comprise a radionuclide of F, I or At and on or more polar groups providing that the compounds can efficiently react with extracellularly located pretargeting vectors and as such used for example for pretargeted cancer diagnostics and cancer therapy.

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Nuclide labelled H-tetrazines and use thereof for PET and SPECT pretargeted imaging and radionuclide therapy

FIELD OF THE INVENTION

5 The present invention is within the field of bioorthogonal chemistry and relates to novel tetrazine compounds for use in pretargeted in vivo imaging and therapy. The compounds are suitable for use in click chemistry, i.e. reactions that join a targeting molecule and a reporter molecule. The compounds comprise a covalently bound radionuclide of F, I or At and are high polar compounds that will not enter cell membranes and are thus particularly useful in relation
10 to cancer diagnostics and cancer therapy using non-internalizing pretargeting vectors.

BACKGROUND OF THE INVENTION

Click chemistry has emerged as a versatile tool for pretargeted imaging, radiotherapy and recently also for specific drug release in vivo. Click chemistry is of particular interest in
15 bioorthogonal chemistry.

Bioorthogonal chemistry refers to any chemical reaction that can occur inside living systems without interfering with native biochemical processes. A pretargeting strategy makes use of bioorthogonal chemistry and proceeds in two steps. A first step is where a substrate is modified with a bioorthogonal functional group (denoted chemical reporter or target vector) and
20 introduced to the patient. Normally, a substrate can be a metabolite, an enzyme inhibitor, monoclonal antibody, nanomedicine, polymer, nanoparticle, etc. The second step is where a probe, that contains the complementary functional group, is introduced and reacts and labels the substrate. The probe is a small effector molecule carrying the label.

Recently, bioorthogonal chemistry has emerged as a versatile tool for nuclear pretargeted
25 imaging of slow accumulating targeting vectors (nanomedicines) such as monoclonal antibodies (mAbs) or other nanomedicines.^[1] Improved target-to-background ratios and lower radiation burden to healthy tissue can be reached using pretargeting compared to conventional targeting strategies. These improved target-to-background ratios are a result of the temporal separation of the slow targeting process of nanomedicines from the actual imaging or
30 therapeutic step. Consequently, the exquisite target specificity of nanomedicines as well as the optimal pharmacokinetics of small molecules for molecular imaging, e.g. rapid target accumulation and blood clearance, can be exploited using pretargeted imaging.^[2, 3] So far, the most prominent reaction for pretargeted imaging is the tetrazine ligation. Excellent chemo selectivity, metabolic stability, high reaction rates and low toxicity make this ligation

exceptional.^[4, 5] The tetrazine ligation itself is based on an Inverse-Electron-Demand Diels-Alder (IEDDA) cycloaddition reaction followed by a retro-Diels-Alder elimination of nitrogen between an electron-deficient tetrazine (Tz) and often a strained trans-cyclooctene (TCO) derivative.^[6, 7] Many efforts have been made in recent years to utilize the tetrazine ligation for
5 pretargeted PET imaging or radionuclide therapy. Initially, TCOs have been used to develop a ¹⁸F-PET pretargeting imaging agent. However, the rapid plasma isomerization of TCOs make them non-ideal.^[2] Thus, the labeling of Tzs gradually emerged as a more feasible approach.^[3]

Positron-Emission-Tomography (PET) and Single-photon emission computed tomography (SPECT) are powerful and routinely used non-invasive imaging tools in precision medicine or
10 drug development.^[4] Its high sensitivity and isotropism are in combination unmatched compared to any other in vivo molecular imaging technique.^[5] Fluorine-18 (¹⁸F) is considered as the “gold standard” PET radionuclide for clinical applications as it provides almost ideal physical characteristics for PET molecular imaging. A relatively short positron range (2.4 mm max. range in water), a good branching ratio (96.7 % positron decay) and a half-life of approx.
15 110 min results in good resolution, a relatively low radiation burden for patients and in the ability to distribute a ¹⁸F-labeled tracer within a several hundred kilometers range.^[12, 13] The PET radionuclide Iodine-124 (¹²⁴I) possesses a half-life of 4.18 d and allows as such for longer timeframes. Iodine-123 (¹²³I) is a standardly used radionuclide for SPECT and is as such useful for SPECT imaging. Recently, targeted radionuclide therapy with alpha-emitters has emerged
20 as a versatile and effective tool to treat cancers, even micrometastases, one of the main reasons of reoccurrence of cancer after initial treatment. Astatine-211 (²¹¹At) is one of the most interesting radionuclide in this respect since it can be simultaneously imaged and per decay only one alpha-particle is emitted and not several as it is the case for many other alpha-emitters such as ²²⁵Ac or ²¹³Bi.

Labeling approaches using radiometals such as ¹¹¹In, ⁶⁴Cu, ⁸⁹Zr, ⁴⁴Sc, ²¹²Pb, ²²⁵Ac, ²¹³Bi or
25 ⁶⁸Ga have been used for pretargeting strategies within the last decade.^[14-17] It would, however, be useful if labeling approaches for other radionuclides such as ¹⁸F, ¹²³I, ¹²⁴I, ¹³¹I and ²¹¹At could be provided. These radionuclides are of particular interest because they are covalently bound to their targeting molecule and allow as such for a bigger flexibility in respect to finetuning the
30 pharmacokinetic profile of the radiopharmaceutical. Size, lipophilicity and rate constants can easily be manipulated. Moreover, from a synthetic point of view, all radionuclides can be introduced from the same or similar precursors and as such, a theranostic pair is easily accessible. A theranostic pair in the context of radiopharmacy allows to accompany a therapy with a diagnosis with the aim of a patient-specific treatment. In this respect, a diagnostic
35 radiopharmaceutical can be used to adequately estimate the effectiveness of a therapeutic

radiopharmaceutical and allows dose estimation and to determine the maximum tolerated dose.

In 2013, the first successful attempt to label a Tz moiety with a covalently bound PET radionuclide, i.e. with carbon-11, was reported by the groups of the present inventors.^[7]

5 Despite the great progresses in the field, no highly reactive ¹⁸F-Tzs were reported until recently, most likely due to the insufficient stability of Tzs during standard aromatic nucleophilic ¹⁸F-fluorination conditions.^[19-22] Only relatively base insensitive and less reactive Tzs with rate constants of approx. 86 M⁻¹ s⁻¹ (measured in 1,4-dioxane, 37 °C) could be radiolabeled via an ¹⁸F-aliphatic substitution (S_N2) strategy. Radiochemical yields (RCYs) up to 18% were
10 reached.^[8] More recently, the preparation of a highly reactive ¹⁸F-labeled glycosylated Tz by Keinänen et al. and an Al[¹⁸F]NOTA-labeled Tz radioligand by Jan-Philip et al. were reported.^[19, 21] The last strategy described exploited the azide-alkyne Huisgen cycloaddition to efficiently label highly reactive Tzs.^[9] However, none of the aforementioned ¹⁸F-labeling procedures seem optimal for clinical applications, since multi-step procedures are usually
15 challenging to set up for clinical routine and Al¹⁸F-labeling procedures are still in its infancy in respect to its scalability.

The most exploited reaction for the synthesis of radiolabeled aryl fluorides is the Nucleophilic Aromatic Substitution reaction (S_NAr). Typically, this type of reaction requires relatively basic conditions and high temperature and as such, they are not ideally applicable to ¹⁸F-label
20 structures containing Tz moieties. ^[20, 23] Recently, however, several milder aromatic ¹⁸F-labeling strategies have been reported that proceed faster, at lower basicity as well as at lower temperatures. Especially, Cu-mediated oxidative fluorination of tin- and boronic ester/acid species ^[24-27], concerted Nucleophilic Aromatic Substitution of uronium or iodonium salts ^[28-30], hypervalent iodonium based precursors ^[11] and minimalistic labeling strategies have proven
25 their potential in this respect.^[30, 32]

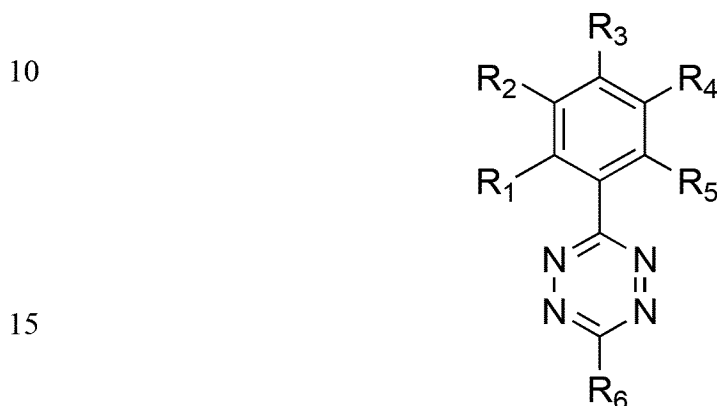
For the above reasons, it would be beneficial to develop a simple, scalable and reliable direct aromatic radionuclide labeling procedure that can be applied to label highly reactive Tzs providing radionuclide-Tz based pretargeted imaging agents with fast reaction kinetics, good metabolic stability and favorable pharmacokinetic profile needed for bioorthogonal chemistry
30 and with the possibility for being provided in clinically relevant amounts.

Iodine labeling and ²¹¹At-radiolabeling of tetrazines have been successfully carried out.^[33-35] None of these tetrazines were applied to pretargeted strategies since their lipophilicity is too high for any reasonable in vivo ligation, at least in any standardly applied pretargeting tumor model.

Thus, a direct radionuclide labeling strategy with radionuclides such as ^{18}F , ^{123}I , ^{124}I , ^{131}I and ^{211}At for highly reactive tetrazines suitable for pretargeted approaches in vivo would be greatly beneficial if not essential for clinical translation of such labeled tetrazines.

5 SUMMARY OF THE INVENTION

Accordingly, in a first aspect, the present invention relates to tetrazine compounds having the following formula I:



Formula I

20 wherein $\text{R}_1\text{-R}_5$ are independently selected from: a radionuclide selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At ; one or more group(s) providing a lipophilicity of $\text{clogD}_{7.4} < -3$ to the compound of Formula I independently selected from the group consisting of a hydroxy group, a sulfonamide, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(\text{O}-\text{CH}_2-\text{CH}_2)_{1-5}-\text{OCH}_2-\text{COOH}$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and
 25 optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl,
 30 (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, a hydroxy group, a

sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine; and wherein R_6 is H;

wherein one of R_1-R_5 is a radionuclide and one or more of the remaining R_1-R_5 is a/group(s) providing a lipophilicity of $\log D_{7.4} < -3$, and any R_1-R_5 remaining thereafter is/are H.

In some embodiments, the radionuclide is ^{18}F , situated at position R_1 , R_2 , R_3 , R_4 or at R_5 .

In other embodiments, the radionuclide is ^{123}I , situated at position R_1 , R_2 , R_3 , R_4 or at R_5 .

In other embodiments, the radionuclide is ^{124}I , situated at position R_1 , R_2 , R_3 , R_4 or at R_5 .

In other embodiments, the radionuclide is ^{131}I situated at position R_1 , R_2 , R_3 , R_4 or at R_5 .

In other embodiments, the radionuclide is ^{211}At situated at position R_1 , R_2 , R_3 , R_4 or at R_5 .

In a preferred embodiment, the radionuclide is situated at position R_4 in Formula I. When situated at position R_4 a faster reaction rate is obtained for providing Formula I. Thus, in one preferred embodiment, the radionuclide is ^{18}F , situated at position R_4 . In another preferred embodiment, the radionuclide is ^{123}I , situated at position R_4 . In another preferred embodiment, the radionuclide is ^{124}I , situated at position R_4 . In another preferred embodiment, the radionuclide is ^{131}I situated at position R_4 . In another preferred embodiment, the radionuclide is ^{211}At situated at position R_4 .

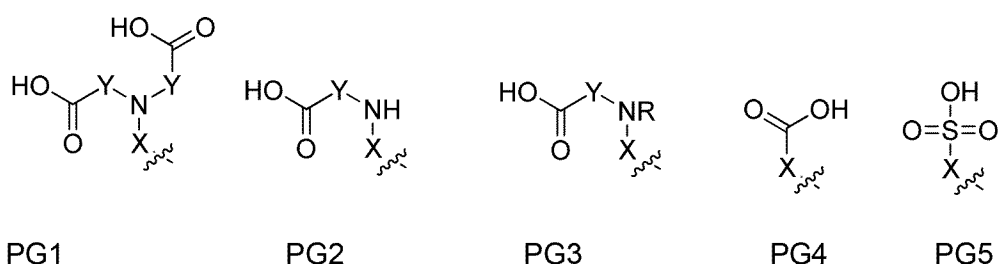
The tetrazine compounds according Formula I comprises at least one group that provides the compounds according to Formula I with a lipophilicity of $\log D_{7.4} < -3$. It has been found that when the tetrazine compounds of Formula I have a sufficiently high polarity, they will reach pretargeting vectors which do not internalize. This is beneficial for their use as imaging agents, as diagnostic agents and as therapeutic agents in relation to certain diseased tissue in animals and humans, such as cancer tissue, cancerous cells, infected cells or pathogens.

The lipophilicity of compounds of formula 1 suited for such purposes was found to have a lipophilicity $\log D_{7.4}$ of approximately less than -3. As shown in Figure 3, the blocking effect increased the lower the lipophilicity value. Thus, the compounds of Formula I must have a lipophilicity $\log D_{7.4}$ of less than -3.

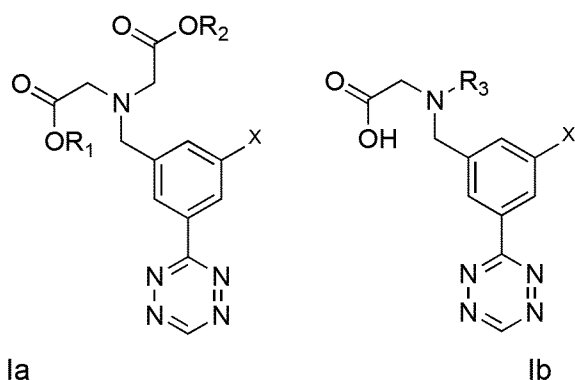
In order for the compounds of Formula I to have a lipophilicity of $\text{clogD}_{7.4} < -3$ or less, one or more of R_1 , R_2 , R_3 , R_4 or R_5 is a group that provides for such a polarity. It has been found that the tetrazines of Formula I will have a $\text{clogD}_{7.4}$ value of -3 or less, when one or more of R_1 , R_2 , R_3 , R_4 or R_5 is selected from $-\text{OH}$, NR_7R_8 , $\text{CH}_2\text{N}(\text{CH}_2\text{COOH})_2$, $\text{CH}_2\text{NHCH}_2\text{COOH}$, $\text{CH}_2\text{NRCH}_2\text{COOH}$, CONR_7R_8 , SO_3H , SO_2NH_2 , and SO_2NH , wherein R is H , CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH , R_7 is H , CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH ; and R_8 is H , CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH .

In a preferred embodiment, one of R_2 or R_4 is $\text{CH}_2\text{N}(\text{CH}_2\text{COOH})_2$, $\text{CH}_2\text{NHCH}_2\text{COOH}$, $\text{CH}_2\text{NRCH}_2\text{COOH}$ wherein R is H , CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH ; while the other is a radionuclide selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At .

In preferred embodiments, the one or more group(s) providing a lipophilicity of $\text{clogD}_{7.4} < -3$ to the compound of Formula I is selected from the polar groups (PG):



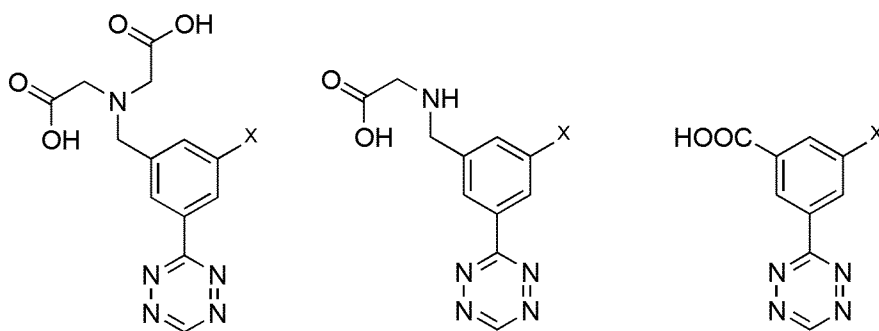
Wherein X is $\text{CO}(\text{CH}_2)_n$, $\text{SO}_2(\text{CH}_2)_n$, $(\text{CH}_2)_n$ or $(\text{OCH}_2\text{CH}_2)_n$ and $n = 0, 1, 2$ or 3 ; and wherein Y is $(\text{CH}_2)_n$, $(\text{OCH}_2\text{CH}_2)_n$ or $\text{CO}(\text{CH}_2)_n$ and $n = 0, 1, 2$ or 3 ; and wherein R is $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOH}$, $(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{COOH}$ and $n = 0, 1, 2$ or 3 ; and wherein the curly sign indicates the link to the aromatic ring. In a preferred embodiment, the compounds of Formula I is selected from the following compounds Ia or Ib:



wherein X is selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At , and R_1 , R_2 and R_3 is independently selected from H, $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOH}$, $(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{COOH}$ and $n = 0, 1, 2$ or 3 .

5

In another preferred embodiment, the compounds of Formula I is selected from the following compounds Ic, Id and Ie:



10

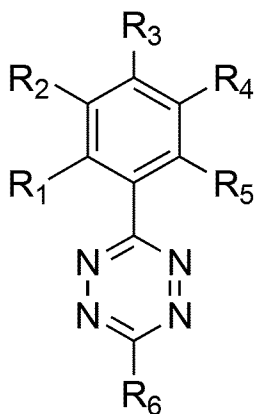
Ic

Id

Ie

wherein X is selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At .

15 In another aspect, the invention provides tetrazine precursors having the general formula II:



20

Formula II

25

wherein one of R_1 - R_5 are independently selected from the group consisting of: SnR_3 , $\text{B}(\text{OR})_2$, I^+-Ar , I double-bonded to R or SiR_3 , wherein R is a linear or branched C1-C6 alkyl, cyclic C1-C6 alkyl, optionally substituted with $-\text{OH}$, $-\text{NH}_2$ or halogen; at least one of the remaining R_1 - R_5 are selected from the group consisting of a substituted amine with 1-5 polyethylene glycol unit(s), a $-(\text{O}-\text{CH}_2-\text{CH}_2)_{1-5}-\text{OCH}_2-\text{COOH}$, optionally substituted heteroaryl, and optionally

30

substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, or an amine, and any R_1-R_5 remaining thereafter is/are H; and

wherein R_6 is a H;

All the precursors of formula II can be used to provide the compounds of formula I. However, some precursors are more suited than others to provide the compound of formula I in high efficiency depending on the specific radionuclide.

In the most preferred embodiments, one of R_1-R_5 in the tetrazine precursor of formula II is SnR_3 or $B(OR)_2$. These precursors are suitable for providing all compounds according to formula I regardless of the specific radionuclide selected.

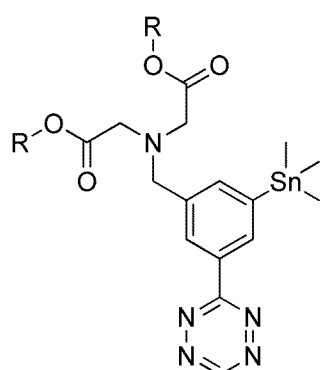
In one preferred embodiment, when one of R_1-R_5 in the precursor of formula II is SiR_3 the radionuclide in the final compound of formula I provided by this precursor is ^{211}At .

In another preferred embodiment, when one of R_1-R_5 in the precursor of formula II is I^+-Ar , I double-bonded to R, the radionuclide in the final compound of formula I provided by this precursor is ^{18}F .

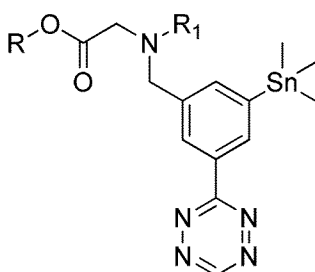
In preferred embodiments, when one of R_1-R_5 in the precursor of formula II is SnR_3 or SiR_3 , R is a linear methyl, ethyl, propyl or butyl and all R's in SnR_3 or SiR_3 , respectively, are the same.

In preferred embodiments the tetrazine precursor of Formula II the SnR_3 , B(OR)_2 , $\text{I}^+\text{-Ar}$, I double-bonded to R ($\text{I}=\text{R}$) or SiR_3 , wherein R is a linear or branched C1-C6 alkyl, cyclic C1-C6 alkyl, optionally substituted with $-\text{OH}$, $-\text{NH}_2$ or halogen is situated at position R_4 . When situated at position R_4 a faster reaction rate is obtained for providing Formula I. Also, the yield of the stannylation reaction and of the labeling are higher, when the SnR_3 , B(OR)_2 , $\text{I}^+\text{-Ar}$, I double-bonded to R ($\text{I}=\text{R}$) or SiR_3 is situated at position R_4 .

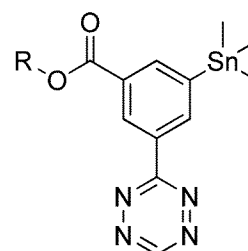
In preferred embodiments, the tetrazine precursor of Formula II is selected from the following structures:



PC1



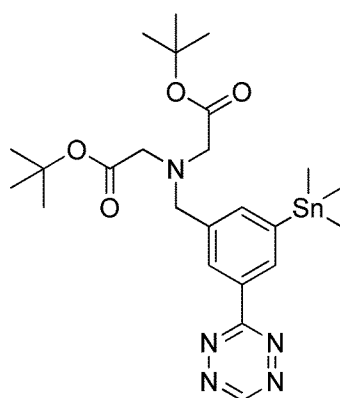
PC2



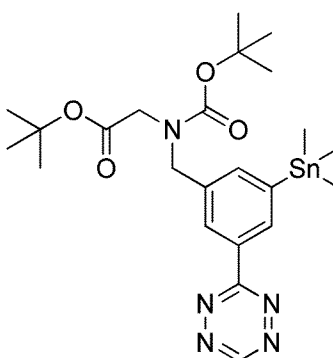
PC3

wherein the protective group R is selected from butyl groups, methylthiomethyl (MTM) groups, tetrahydropyranyl (THP) groups or benzyloxymethyl (BOM) esters, and R_1 is selected from a Boc group, trityl group, acetamide, carbamate group, $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOtBu}$, $(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CO}(\text{CH}_2)_n\text{COOtBu}$ wherein $n = 0, 1, 2$ or 3 .

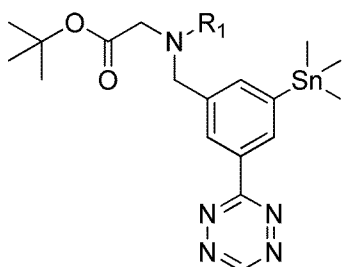
In another preferred embodiment, the tetrazine precursor of Formula II is selected from the following structures:



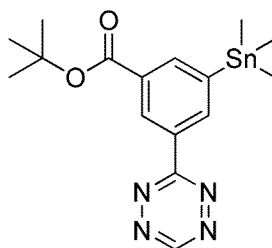
PC4



PC5



PC6



PC7

- 5 wherein R_1 is selected from a Boc group, trityl group, acetamide, carbamate group, $(CH_2)_nCH_3$, $(OCH_2CH_2)_nOH$, $CO(CH_2)_nCOOtBu$, or $(CH_2CH_2O)_nCH_2CO(CH_2)_nCOOtBu$ wherein $n = 0, 1, 2$ or 3.

As shown herein, the tetrazine compounds of Formula I A compound are excellent for use in
 10 bioorthogonal chemistry. All if the tetrazines of Formula I tested showed a TCO click ability close to 100%.

It is moreover shown in the present Examples, that the compounds of Formula I provides very good imaging results in mice being infested with a human cancer type as shown with PET scanning. The applicability of the tetrazine compounds of Formula I in various imaging
 15 techniques such as PET and SPECT will depend on the specific radionuclide selected.

Accordingly, in one aspect of the invention, the compounds of Formula I is for use in biorthogonal chemistry for pretargeted strategies.

20 In another aspect of the invention, the compounds of Formula I is for use in diagnostics. In a preferred embodiment, the diagnostics is cancer diagnostics. The cancer can be any kind of cancer.

In one aspect of the invention, the radionuclide of the tetrazine derivative of Formula I is ^{18}F or
 25 ^{124}I use in PET imaging.

In one aspect of the invention, the radionuclide of the tetrazine derivative of Formula I is ^{123}I for use in SPECT imaging.

30 In a preferred embodiment, the PET or SPECT imaging according to the above is of cancer tissue.

In one aspect of the invention, the radionuclide of the tetrazine derivative of Formula I is ^{211}At or ^{131}I for use in radionuclide therapy.

- 5 In a preferred embodiment of that aspect, the radionuclide therapy is of a cancer disease.

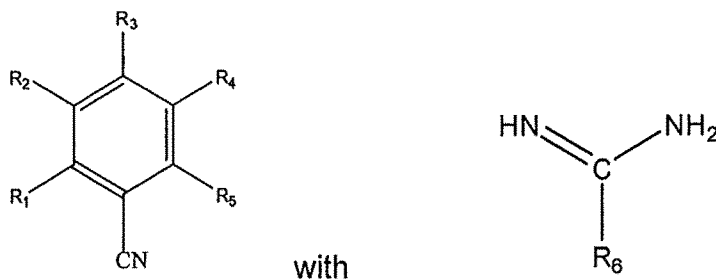
In another preferred embodiment of that aspect, the radionuclide therapy is applied to kill extracellular pathogens or any kind of unwanted cell, virus, microorganism which is extracellularly deposited.

10

Due to the one or more polar groups in Formula I, these compounds can be used in imaging, diagnostics and therapy wherein it is desired that the compound does not penetrate cell membranes.

- 15 Another aspect of the invention provides a method for preparing a compound of Formula I as detailed in general procedure a, b, c and d.

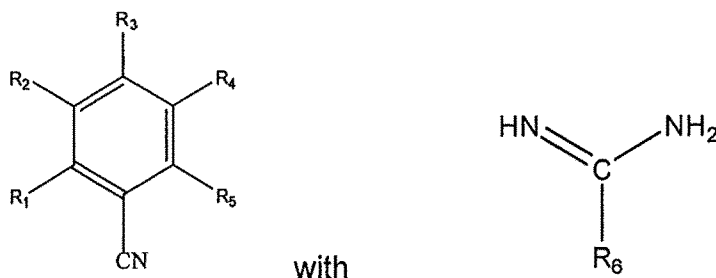
In one embodiment the method comprises reacting



- 20 wherein one of R_1 - R_5 are independently selected from the group consisting of: SnR_3 , B(OR)_2 , I^+-Ar , I double-bonded to R or SiR_3 , wherein R is a linear or branched C1-C6 alkyl, cyclic C1-C6 alkyl, optionally substituted with $-\text{OH}$, $-\text{NH}_2$ or halogen; at least one of the remaining R_1 - R_5 groups are selected from one or more group(s) selected from the group consisting
- 25 of a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(\text{O}-\text{CH}_2-\text{CH}_2)_{1-5}-\text{OCH}_2-\text{COOH}$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-

C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine; with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, or an amine; and any R_1-R_5 remaining thereafter is/are H; and wherein R_6 is a H;

15 In another preferred embodiment, the method comprises reacting



wherein one of R_1-R_5 are independently selected from I or F and, at least one of the remaining R_1-R_5 are selected from the group consisting of a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl;

wherein optionally substituted means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine; and any R_1-R_5 remaining thereafter is/are H; and wherein R_6 is a H.

In a preferred embodiment, the method is carried out at a temperature range of from 50° to 70°C.

In another preferred embodiment, the method is carried out by adding water after cooling to room temperature followed by addition of HCl and extraction with EtOAc.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Scheme showing the principle of labelling tetrazines with ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At

Figure 2 Scheme showing labeling of Tz-1 as a model compound and table with optimization data

Figure 3 Scheme showing the ability of ^{19}F -tetrazines to block ^{111}In -DOTA-tetrazine (Q)

Figure 4 Structure of compound ^{18}F -UB108, ^{18}F -UB137 and ^{111}In -DOTA-tetrazine (Q)

Figure 5 Scheme showing the synthesis UB108 and UB137

Figure 6 Synthesis of the tin-precursor of UB108 and UB137 and radiolabeling of ^{18}F -UB108 and ^{18}F -UB137

Figure 7 NMR of UB108

Figure 7a NMR of UB108 precursor (UB161)

Figure 8 NMR of UB137

Figure 8a NMR of UB137 precursor (UB266)

Figure 9 HPLC of reference compound UB108 and radio-HPLC of purified ^{18}F -UB108.

Figure 10 HPLC of reference compound UB108 and radio-HPLC of purified ^{18}F -UB137.

Figure 11 UB-108 click-ability to a TCO derivative

Figure 11a UB-137 click-ability to a TCO derivative

Figure 12 PET scan and biodistribution of ^{18}F -UB108 one 1h after injection of pretreated tumor bearing mice with TCO-CC49 three days before ^{18}F -UB108 injection

Figure 13 PET scan and biodistribution of ^{18}F -UB137 one 1h after injection of pretreated tumor bearing mice with TCO-CC49 three days before ^{18}F -UB137 injection.

Figure 14 PET/CT scan of CC49-TCO pretargeted [^{18}F]-UB108 in LS174T tumor xenograft bearing mice.

Figure 15 Structural scaffolds for the ligation with TCO, of Tz-derivatives.

Figure 16 Results from the blocking assay. (A) Schematic display of the blocking assay. (B)

5 Blocking effect of non-radiolabeled Tz determined as the change in tumor uptake of [^{111}In]Q 22 h p.i., (C) and (D) Correlation of blocking effect and $\text{clogD}_{7.4}$ for Tz-derivatives with similar IEDDA reactivity.

Figure 17 schematic view of the Cu-mediated click-radiolabeling for the synthesis of ^{18}F -labeled Tz-probes, the structure of the TZ-probes tested in example 140.

10 Figure 18 (A) Schematic display of the blocking assay, (B) tumor uptake (C) T/M ratio, (D) blocking effect, and (E) Pretargeted PET imaging in BALB/c nude mice bearing LS174T tumor xenografts with six ^{18}F -labeled Tz-derivatives.

DETAILED DESCRIPTION OF THE INVENTION

15 The present invention provides tetrazine compounds labelled with a ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At radionuclide and having one or more polar groups that provide a H-tetrazine compound with a lipophilicity of $\text{clogD}_{7.4} < -3$. This lipophilicity is needed to obtain reasonable tumor accumulation (normalized blocking effect > 70), in standardly applied tumor models for pretargeted strategies. The lipophilicity of a compound can be found by use of a software for

20 calculating the lipophilicity based on the chemical composition and structure of the compound. Even though the lipophilicity should approximately be the same for a given compound regardless of the method used for determining the lipophilicity, deviations may occur between methods. In the context of the present invention, the lipophilicity value clogD at physiological pH (7.4) were calculated using the software "Chemicalize", 2019, from ChemAxon.

25 A simple, scalable and reliable direct labeling method for labeling tetrazines with the ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At is also provided together with tin- or boronic species precursors or silyl, iodonium or ylide precursors, especially for ^{211}At labeling. The tetrazine compounds of the invention are shown to be suitable for use in biorthogonal chemistry including imaging-based diagnostics, such as PET and SPECT and in radionuclide therapy. Due to the high polarity of

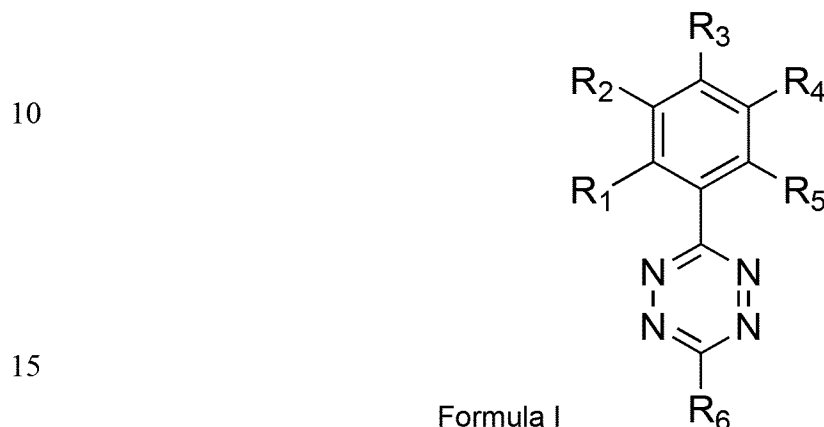
30 the tetrazine compounds disclosed herein, the tetrazine compounds cannot penetrate cell membranes and are thus particularly suitable for pretargeted strategies of non-internalizing pretargeting vectors. The tetrazine compounds of the invention display characteristics in mice that qualifies their use in one or more of PET imaging, SPECT imaging and radionuclide therapy in humans.

35

The H-tetrazine compounds labelled with a ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At radionuclide and having one or more polar groups that provide the tetrazine compound with a lipophilicity of $\text{clogD}_{7.4} < -0.5$. The compounds as disclosed herein were prepared from tin- and boronic precursors or silyl, iodonium or ylide precursors and utilized to label tetrazines with ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At .

5 The principle of the labelling is shown in Figure 1.

In the context of the present invention, an "H-tetrazine" is defined as:



wherein one of $\text{R}_1\text{-R}_5$ is a radionuclide selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At ; and
wherein one or more $\text{R}_1\text{-R}_5$ is a group providing a lipophilicity of $\text{clogD}_{7.4} < -3$ to the compound
20 of Formula I; and wherein the remaining $\text{R}_1\text{-R}_5$ are H; and R_6 H.

The "H" in H-tetrazine refers to the H at position R_6 in formula I.

It was found that compounds of formula I having a high polarity as determined by their
25 lipophilicity value $\text{clogD}_{7.4}$ (Figure 3) provide high blocking effects in tumor blocking assays (Fig 3). These compounds have rate constants of approximately $50.000\text{-}90.000 \text{ M}^{-1} \text{ s}^{-1}$ (determined with standard TCO at 37°C in PBS).

Tz 1 (Figure 2) was selected as a model compound for ^{18}F -labeling, due its easy accessibility
30 and good stability against hard bases. Precursors and reference compounds were synthesized as described in Examples 1 to 7. It was found that commonly used ^{18}F -labeling strategies resulted in decomposition products. On the contrary, for the Cu-mediated radionuclide reaction through the stannate precursor (Figure 2), labeling attempts resulted in a radiochemical conversion (RCC) of approximately 20%. In a next step, we optimized the RCC of the Cu-mediated radionuclide. Parameters such as temperature, time and base amount were
35 screened and led to a further RCC increase of circa 10% (Figure 2).

It was then studied if the identified labeling conditions could be applied to more reactive Tzs. In this respect, Tzs with stepwise increased reactivity were chosen and tested to investigate the product scope of the suggested labeling procedure. Precursors and reference were synthesized using known procedures [35-38] and radiolabeling was conducted using the best conditions identified for our model compound. Moderate RCCs (10-30%) as well as sufficient decay-corrected (d.c.) RCYs (10-24%) could be isolated at the end of synthesis (EOS) for methyl-, phenyl- and H-Tzs. The automated synthesis including [¹⁸F]fluoride collection, azeotropic drying, labeling and HPLC separation was carried out within 90 minutes (Example 9). Radiochemical purity (RCP) and molar activity (Am) were good and within the area that we usually observe for other tracers. A typical activity yield was 215 MBq starting from 1.60 GBq fluoride-18. As expected, high reactive Tz resulted in the lowest RCY. However, observed RCYs are in the range of many clinically applied PET tracers. Table 1, annexed to this description, displays the found trend.

To study the effect of different substituents, [¹⁸F]-9 (Figure 4) was selected for further analysis since it displayed the highest reaction rate, which is one of the most crucial factors for pretargeted in vivo applications. In this respect, the substitution pattern was correlated with its synthetic accessibility and RCCs (as a predictor for RCYs). Necessary iodine Tz intermediates were obtained from the corresponding nitriles. 6-substituted stannate analogues could only be synthesized as methyl or methoxy derivatives. Similar stannate precursor formation at the 4-position succeeded only for the methoxy analogue. Both observations can most likely be explained by steric hindrance effects.^[24-26] As such, a 3,5-disubstitution pattern is best suited for oxidative ¹⁸F-fluorination.

It is demonstrated herein that success of pretargeted imaging is strongly dependent on the polarity and the rate constant of the applied Tz (Figure 3). Low polarity (a lipophilicity of $\text{clogD}_{7.4} < -3$) and rate constants between 50.000-90.000 M⁻¹s⁻¹ measured in PBS and using TCO resulted in target-to-background ratios (Figure 3). In this respect, we designed two highly reactive Tzs, which allowed ¹⁸F-labeling and respective introduction of polar groups (Figure 4-8). UB137 and UB108 were synthesized in sufficient yields via a Pinner-like synthesis as described in Example 97 and 106 and their ability to be used in pretargeted imaging were evaluated. The results were compared with the performance of compounds UB-65 and RGV-52 (Figure 3). Efficiency in vivo was tested in an ex vivo screening assay lately published by our group and is inspired by traditional receptor blocking studies. In short, tumour-bearing mice are injected with a validated TCO-modified pretargeting vector 72h before a test ¹⁹F-Tz is administered. Subsequently after 2 hours, a pretargeted established ¹¹¹In-DOTA-Tz (Q)^[6] is

administered to the same mouse. An ex vivo biodistribution study is subsequently conducted 22h later. In this manner, the blocking ability of the cold ^{19}F -Tz can be evaluated. Higher blocking capacity is correlated with better in vivo imaging performance of the respective Tz. Polar Tz's, UB108 and UB137 resulted in the best blocking effect (Figure 3) and was as such selected for further development. The in vitro stability of UB108 and UB137 was assessed by analytical HPLC in Phosphate-buffered saline (PBS), Example 13 for UB108. UB108 and UB137 did not show degradation in PBS after 12 h at 37 °C. Consequently, the organotin precursors were synthesized with an unoptimized synthetic strategy (Figure 6, Examples 101 and 110). Radiolabeling succeeded in a one-pot, two step sequence with a RCY (d.c.) of approx. 10% and an overall synthesis time of ca. 90 minutes including synthesis, separation and formulation steps. An activity yield of 636 MBq starting from 11 GBq fluoride-18, and RCP of 98% at EOS was observed (Figure 6 and 11). UB137 could be labeled with similar results (Figure 6 and 7). The tetrazine ligation of ^{18}F -UB108 with s-TCO occurred rapidly, forming ligation products after click reaction (Figure 11). Stability evaluation was completed for the formulated compound ^{18}F -UB108 in PBS at room temperature. These results showed no significant decomposition after 4 h post-injection and importantly maintained their reactivity with TCO at all the time points tested (96% recovery). UB137 could be labeled with similar results.

To test the tetrazine ligation in living animals, we administered CC49-TCO to mice bearing colon cancer xenografts overexpressing TAG72, followed by the injection of ^{18}F -UB108 or ^{18}F -UB137 two days later. We selected the TAG72 antigen as it is overexpressed in a wide range of solid tumours, including, colorectal cancer and its limited internalization and as CC49 binds to the target and is not internalized afterwards. This pretargeting model is considered as the "Gold standard".^[15, 39] Figure 12 and 13 displays the gained image 1h after ^{18}F -UB108 or ^{18}F -UB137 administration. The contrast is thus far the highest observed of all tetrazines after 1 h.

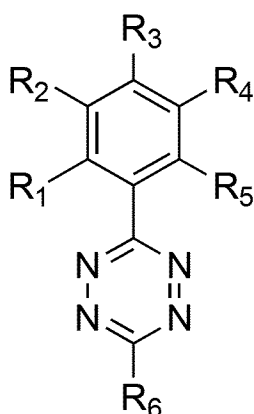
We further describe the a pretargeted blocking assay that allows for the investigation of the *in vivo* fate of a structurally diverse library of 45 unlabeled tetrazines and their capability to reach and react with pretargeted trans-cyclooctene (TCO)-modified antibodies in tumor-bearing mice (cf. example 140). This study enables the assessment of the correlation of click reactivity and lipophilicity of tetrazines with their *in vivo* performance. High-rate constants ($>50,000 \text{ M}^{-1}\text{s}^{-1}$) for the reaction with TCO and low calculated $\log D_{7.4}$ values (below -3) of the tetrazine were identified as strong indicators for successful pretargeting. Radiolabeling gave access to a set of selected ^{18}F -labeled tetrazines, including highly reactive scaffolds, which were used in pretargeted PET imaging studies to confirm the results from the blocking study. These insights

thus enable the rational design of tetrazine probes for *in vivo* application and will thereby assist the clinical translation of bioorthogonal pretargeting.

In conclusion, this work showed the first ^{18}F -direct labeling strategy of highly reactive and polar Tzs, starting from organotin precursors via a Cu-mediated approach. This strategy allows us to synthesize and radiolabel two promising ^{18}F -Tz, [^{18}F]-UB108 and [^{18}F]-UB137, as a tetrazine ligation based radiotracer for pretargeted *in vivo* imaging. The developed procedure is simple, short, reproducible as well as scalable and as such, superior to previously used ^{18}F -multistep labeling strategies with regard to clinical applications. The compounds surprisingly result in very good target-to-background ratios already 1 h after injection unreached with any other tetrazines thus far. Based on the shown correlation between low calculated $\log D_{7.4}$ values (below -3) of the tetrazine and the applicability in successful pretargeting, the applicability of the herein provided tetrazines *in vivo* is proven.

The invention accordingly comprises the following items:

1. A H-tetrazine compound, having the following formula I:



Formula I

wherein R_1 - R_5 are independently selected from: a radionuclide selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At ; one or more group(s) providing a lipophilicity of $\log D_{7.4} < -3$ to the compound of Formula I independently selected from the group consisting of a hydroxy group, a sulfonamide, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(\text{O}-\text{CH}_2-\text{CH}_2)_{1-5}-\text{OCH}_2-\text{COOH}$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10) alkenyl, (C2-

C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine; and wherein R₆ is H;

wherein one of R₁-R₅ is a radionuclide and one or more of the remaining R₁-R₅ is a/are group(s) providing a lipophilicity of $\log D_{7.4} < -3$, and any R₁-R₅ remaining thereafter is/are H.

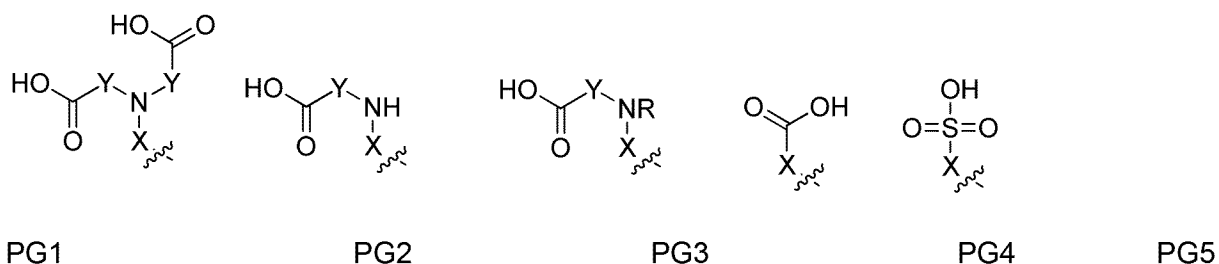
2. A compound according to item 1, wherein said one or more group(s) providing a lipophilicity of $\log D_{7.4} < -3$ to the compound of Formula I; provides a lipophilicity of $\log D_{7.4} < -3$ such as $\log D_{7.4} < -6$.

3. A compound according to any of the preceding items, wherein said one or more group(s) providing a lipophilicity of $\log D_{7.4} < -3$ to the compound of Formula I is selected from: -OH, NR₇R₈, CH₂N(CH₂COOH)₂, CH₂NHCH₂COOH, CH₂NRCH₂COOH, CONR₇R₈, SO₃H, SO₂NH₂, and SO₂NH, wherein R is H, CH₃, CH₂CH₃, CH₂CH₂CH₃ or CH₂COOH, R₇ is H, CH₃, CH₂CH₃, CH₂CH₂CH₃ or CH₂COOH; and R₈ is H, CH₃, CH₂CH₃, CH₂CH₂CH₃ or CH₂COOH.

4. A compound according to any of the preceding items, wherein said radionuclide is situated at position R₄ in formula I.

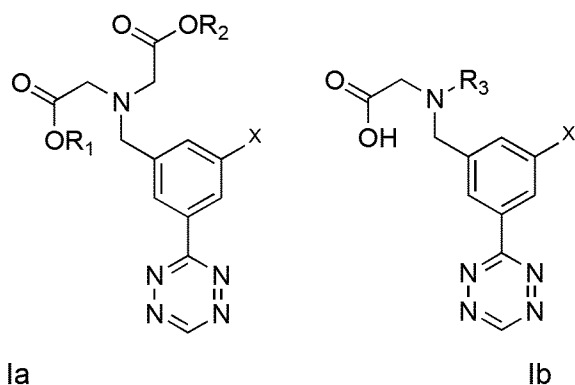
5. A compound according to items 1 to 3, wherein said group providing lipophilicity of $\log D_{7.4} < -3$ is selected from CH₂N(CH₂COOH)₂, CH₂NHCH₂COOH, CH₂NRCH₂COOH, wherein R is H, CH₃, CH₂CH₃, CH₂CH₂CH₃ or CH₂COOH, and is situated at position R₂ or R₄ in formula I; and wherein said radionuclide is situated at the other position R₂ or R₄ in Formula I.

6. A compound according to any of the preceding items, wherein said group providing a lipophilicity of $\text{clogD}_{7.4} < -3$ to the compound of Formula I is selected from the polar groups (PG):



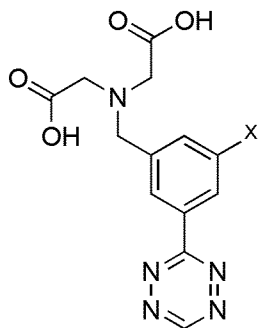
wherein X is $\text{CO}(\text{CH}_2)_n$, $\text{SO}_2(\text{CH}_2)_n$, $(\text{CH}_2)_n$ or $(\text{OCH}_2\text{CH}_2)_n$ and $n = 0, 1, 2$ or 3 ; and wherein Y is $(\text{CH}_2)_n$, $(\text{OCH}_2\text{CH}_2)_n$ or $\text{CO}(\text{CH}_2)_n$ and $n = 0, 1, 2$ or 3 ; and wherein R is $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOH}$, $(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{COOH}$ and $n = 0, 1, 2$ or 3 ; and wherein the curly sign indicates the link to the aromatic ring.

7. A compound according to item 1 selected from:

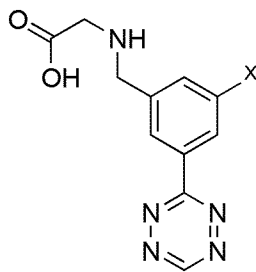


wherein X is selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At , and R_1 , R_2 and R_3 is independently selected from H, $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOH}$, $(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{COOH}$ and $n = 0, 1, 2$ or 3 .

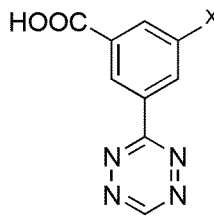
8. A tetrazine according to item 1 selected from:



Ic



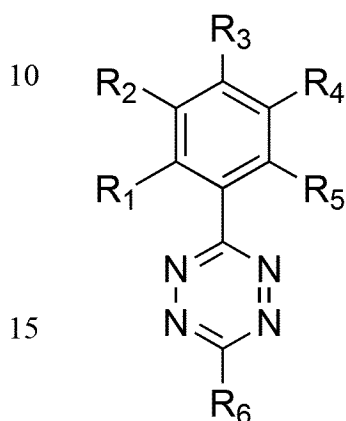
Id



Ie

5 wherein X is selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At .

9. A tetrazine precursor having the following formula II:



Formula II

20 wherein one of R₁-R₅ are independently selected from the group consisting of: SnR₃, B(OR)₂, I⁺-Ar, I double-bonded to R or SiR₃, wherein R is a linear or branched C₁-C₆ alkyl, cyclic C₁-C₆ alkyl, optionally substituted with -OH, -NH₂ or halogen; at least one of the remaining R₁-R₅ are selected from one or more group(s) consisting of substituted amine with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, optionally substituted heteroaryl, and
 25 optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine (C₁-C₁₀) alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, (C₁-C₁₀)alkylene, (C₁-C₁₀)alkoxy, (C₂-C₁₀)dialkylamino, (C₁-C₁₀)alkylthio, (C₂-C₁₀)heteroalkyl, (C₂-C₁₀)heteroalkylene, (C₃-C₁₀)cycloalkyl, (C₃-C₁₀)heterocycloalkyl,
 30 (C₃-C₁₀)cycloalkylene, (C₃-C₁₀)heterocycloalkylene, (C₁-C₁₀)haloalkyl, (C₁-

C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, or H; and wherein R₆ is a H.

10. A tetrazine precursor according to item 9, wherein one of R₁-R₅ is selected from SnR₃ or B(OR)₂.

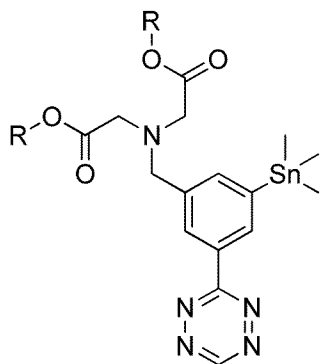
11. A tetrazine precursor according to item 9, wherein one of R₁-R₅ is selected from SnR₃ or SiR₃ wherein R is a linear methyl, ethyl, propyl or butyl and all R's are the same.

12. A tetrazine precursor according to items 9 or 11, wherein one of R₁-R₅ is SiR₃ and wherein the radionuclide in the final compound of formula I provided by this precursor is ²¹¹At.

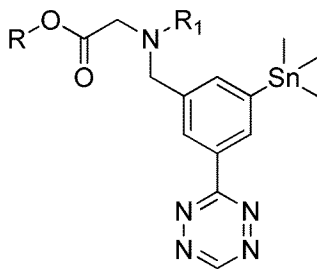
13. A tetrazine precursor according to item 9, wherein one of R₁-R₅ is I+-Ar, I double-bonded to R, and wherein the radionuclide in the final compound of formula I provided by this precursor is ¹⁸F.

14. A tetrazine precursor according to any of items 9 to 13 wherein said group selected from SnR₃, B(OR)₂, I+-Ar, I double-bonded to R or SiR₃, is situated at position R₄ in Formula II.

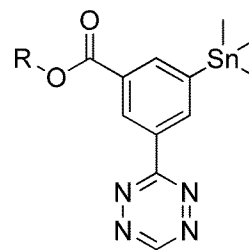
15. A tetrazine precursor according to item 9 selected from:



PC1



PC2

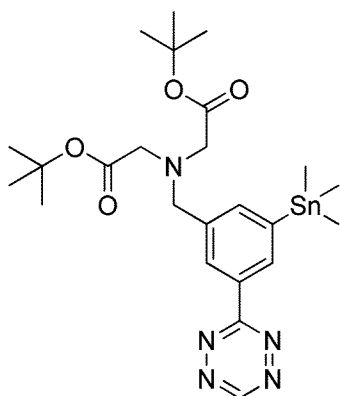


PC3

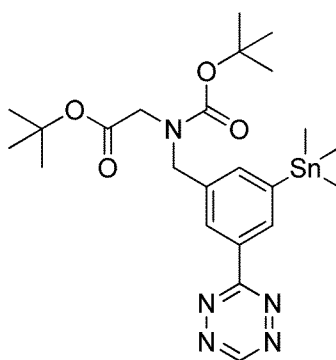
wherein the protective group R is selected from butyl groups, methylthiomethyl (MTM) groups, tetrahydropyranyl (THP) groups or benzyloxymethyl (BOM) esters, and R1 is selected from a

5 Boc group, trityl group, acetamide, carbamate group, $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOtBu}$, $(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CO}(\text{CH}_2)_n\text{COOtBu}$ wherein $n = 0, 1, 2$ or 3 .

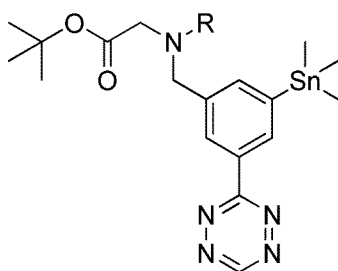
16. A tetrazine precursor according to item 9 selected from:



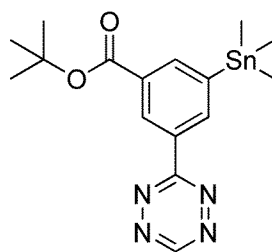
PC4



PC5



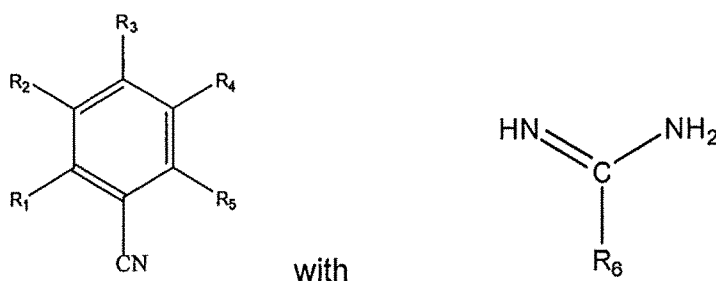
PC6



PC7

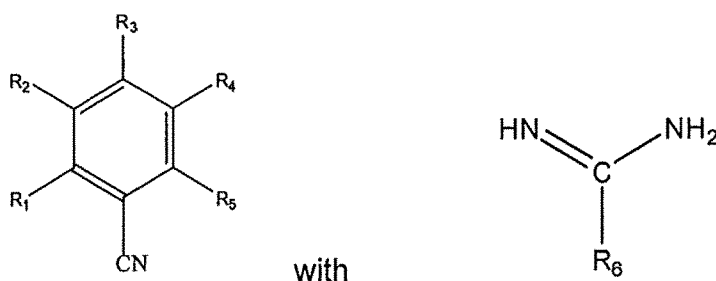
wherein R is selected from a Boc group, trityl group, acetamide, carbamate group, $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOtBu}$, or $(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CO}(\text{CH}_2)_n\text{COOtBu}$ wherein $n = 0, 1, 2$ or 3 .

- 5 17. A compound as defined in any of items 1-8 for use in bioorthogonal chemistry.
18. A compound as defined in any of items 1-8 for use in diagnostics.
19. A compound according to item 18, wherein said diagnostics is cancer diagnostics
- 10 20. A compound as defined in any of items 1-8 wherein the radionuclide is ^{18}F or ^{124}I use in PET imaging.
21. A compound as defined in any of items 1-8 wherein the radionuclide is ^{123}I for
- 15 use in SPECT imaging.
22. A compound for use as defined in any of items 20-21, wherein the imaging is of cancer tissue
- 20 23. A compound as defined in any of items 1-8 wherein the radionuclide is ^{211}At or ^{131}I for use in radionuclide therapy.
24. A compound for use as defined in item 23, wherein the radionuclide therapy is of a cancer disease.
- 25 25. A compound for use as defined in item 24, wherein the radionuclide therapy is applied to target vectors which do not internalize, such as pathogen targets and/or cells infected with pathogens.
- 30 26. A compound for use as defined in any of items 17-25, wherein the compound does not penetrate cell membranes.
27. A method comprising reacting



wherein one of R₁-R₅ are independently selected from the group consisting of: SnR₃, B(OR)₂, I⁺-Ar, I double-bonded to R or SiR₃, wherein R is a linear or branched C1-C6 alkyl, cyclic C1-C6 alkyl, optionally substituted with -OH, -NH₂ or halogen; and the remaining R₁-R₅ are selected from the group consisting of a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, an substituted amine; with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, or H; and wherein R₆ is a H.

28. A method according to item 27 comprising reacting



wherein one of R₁-R₅ are independently selected from I or F and the remaining R₁-R₅ are selected from the group consisting of a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10) alkenyl, (C2-C10) alkynyl, (C1-C10) alkylene, (C1-C10) alkoxy, (C2-C10) dialkylamino, (C1-C10) alkylthio, (C2-C10) heteroalkyl, (C2-C10) heteroalkylene, (C3-C10) cycloalkyl, (C3-C10) heterocycloalkyl, (C3-C10) cycloalkylene, (C3-C10) heterocycloalkylene, (C1-C10) haloalkyl, (C1-C10) perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6) alkyloxy-(C1-C4) alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, or H; and wherein R₆ is a H.

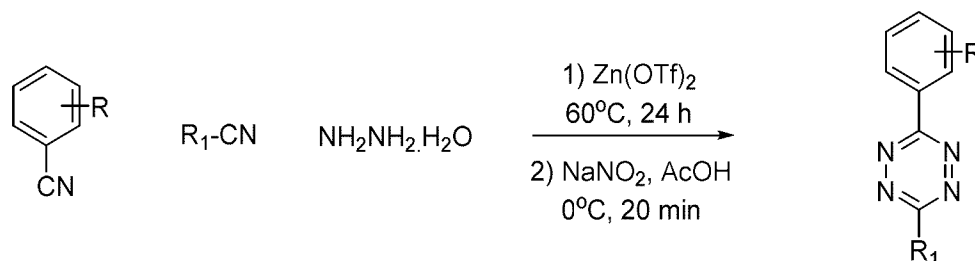
29. A method according to item 27 or 28, wherein the reaction is carried out at a temperature range of from 50 to 70 °C.

30. A method according to any of items 27 to 29, wherein water is added after cooling to room temperature followed by addition of HCl and extraction with EtOAc.

EXAMPLES

General Procedures

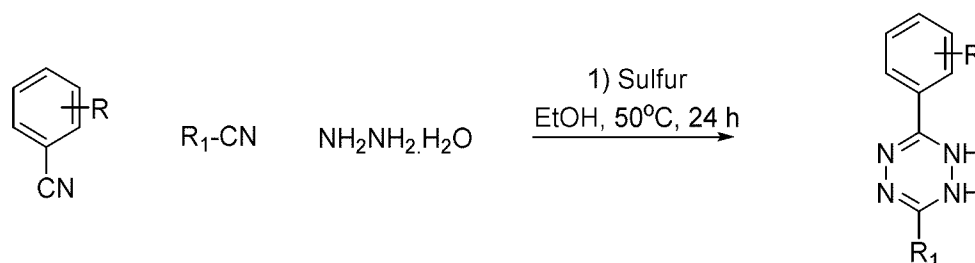
General Procedure A. Synthesis of 3- substituted -6-substituted-1,2,4,5-tetrazine.



- 5 The preparation of this intermediates, were performed using a method described previously.^[13] The selected aromatic halogenated nitrile (1 mmol, 1 equiv.), $\text{Zn}(\text{OTf})_2$ (182 mg, 0.50 mmol, 0.5 equiv.) and hydrazine monohydrate (2.43 mL, 50 mmol, 50 equiv.), along with the appropriate second nitrile (5 mmol, 5 equiv.), were added to a microwave vial equipped with a stir bar and sealed. The reaction was allowed to stir at 60°C for 24 hours before being allowed
- 10 to cool to room temperature and unsealed. NaNO_2 (1.35 g, 20 mmol, 20 equiv.) in water (30 mL) was added to the now yellow mixture followed by dropwise addition of acetic acid (14 mL), producing a mixture red in colour. The mixture was then extracted with EtOAc, washed with brine, dried over MgSO_4 , filtered, and concentrated under reduced pressure. The tetrazine was then purified via automatic flash chromatography utilising in various mixtures as the eluent.

15

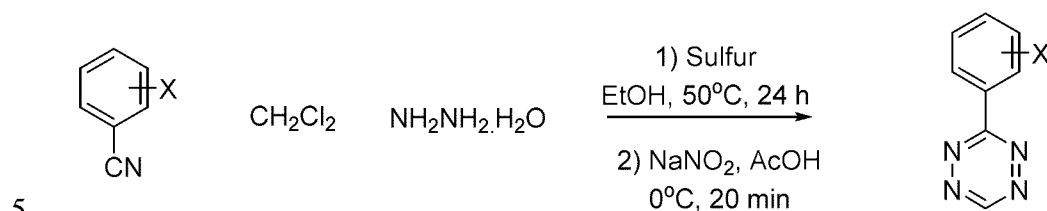
General Procedure B. Synthesis of 3- substituted -6-substituted-1,2-dihydro-1,2,4,5-tetrazine



- The preparation of this intermediates, was performed using a method described previously.^[15] The selected aromatic halogenated nitrile (1 mmol, 1 equiv.), sulfur (513 mg, 2.00 mmol, 2 equiv.), hydrazine monohydrate (804 μL , 16.5 mmol, 16.5 equiv.) and ethanol (2.0 mL), along with the appropriate second nitrile (4.5 mmol, 4.5 equiv.), were added to a microwave vial equipped with a stir bar and sealed. The reaction mixture was heated to 125°C for 2 hours before being allowed to cool to room temperature, unsealed and dry under vacuum. The mixture was suspended in 10 mL water and extracted with CH_2Cl_2 (2x10 mL), washed with
- 20

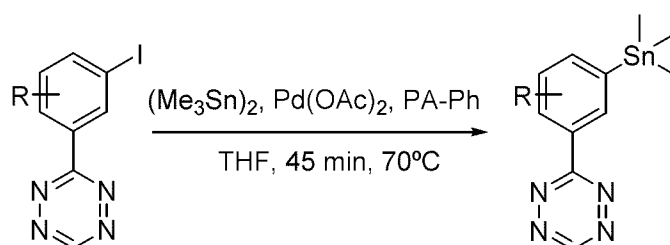
brine, dried over MgSO_4 , filtered and concentrated under reduced pressure. The tetrazine was then purified via automatic flash chromatography utilising in various mixtures as the eluent.

General Procedure C. Synthesis of 3-substituted-6-H-1,2,4,5-tetrazine



The preparation of this intermediates, was performed using a method described previously.^[15] CH_2Cl_2 (0.256 mL, 4.00 mmol, 1 equiv.), sulfur (0.257 g, 1.00 mmol, 0.25 equiv.), hydrazine monohydrate (1.6 mL, 32.00 mmol, 8 equiv.) and ethanol (4.0 mL) along with the appropriate nitrile (4 mmol, 1 equiv.) were added to a microwave vial equipped with a stir bar. The vessel was sealed, and the reaction mixture was heated to 50 °C for 24 hours, before being allowed to cool to room temperature and unsealed. Then 3 ml of CH_2Cl_2 and NaNO_2 (2.8 g, 40.00 mmol, 10 equiv.) in water (40 ml) were added to the now yellow mixture followed by dropwise addition of acetic acid (14 mL), producing a mixture red in colour. The reaction mixture was extracted with CH_2Cl_2 , washed with brine, dried with MgSO_4 and filtered before concentrating *in vacuo*. The tetrazine was then purified via flash chromatography utilising n-Heptane and EtOAc in various mixtures as the eluent and recrystallized in n-Heptane.

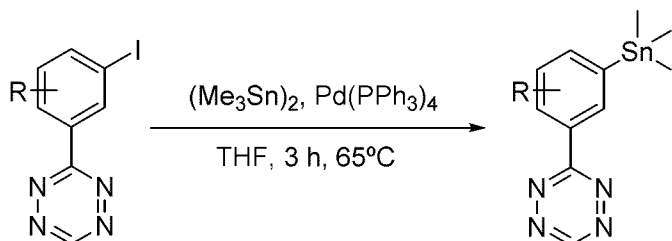
General Procedure D.1. Synthesis of organotin compounds



The preparation of these intermediates, was performed using a method described previously with minor modifications.^[14] Palladium acetate (4.5 mg, 12%) and 1,3,5,7-tetramethyl-2,4,8-trioxa-(2,4-dimethoxyphenyl)-6-phosphaadamantane (PA-Ph) (9.8mg, 20%) dry THF (1.5 mL) and hexamethylditin (75 μL , 137 mg, 0.42 mmol, 2.5 equiv.) were successively added to a microwave vial equipped with a stir bar which was then sealed and purged with N_2 . A solution of the appropriate *iodo-phenyl-1,2,4,5-tetrazine* (0.17 mmol) in dry THF (1 mL) was added via a syringe and the reaction allowed to stir at 70 °C in a microwave for 45 minutes. The reaction was allowed to cool to room temperature and unsealed before being quenched with saturated

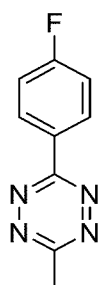
aqueous KF (1 mL). The solution was extracted with CH₂Cl₂ washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. The tetrazine was then purified via automatic flash chromatography utilising n-Heptane and EtOAc as the eluent.

5 General Procedure D.2. Synthesis of organotin compounds.



The preparation of these intermediates, was performed using a method described previously with minor modifications.^[14] Pd(PPh₃)₄ (19.4 mg, 10%) and Hexamethylditin (87 µL, 0.42 mmol, 2.5 equiv.) were successively added to a microwave vial equipped with a stir bar which was then sealed and purged with N₂. A solution of the appropriate *iodo-phenyl-1,2,4,5-tetrazine* (0.17 mmol) in dry THF (2.5 mL) was added via a syringe and the reaction allowed to stir at 65 °C in a microwave for 3 hours. The reaction was allowed to cool to room temperature and unsealed before being quenched with saturated aqueous KF (1 mL). The solution was extracted with CH₂Cl₂ washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. The tetrazine was then purified via automatic flash chromatography utilising n-Heptane and EtOAc as eluent.

Example 1

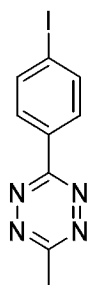


20 **3-(4-fluorophenyl)-6-methyl-1,2,4,5-tetrazine (RGV_48, Tz-1):** The final compound was obtained from 4-fluorobenzonitrile (121 mg, 1.00 mmol) and acetonitrile (261 µL, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.57 g (30%) of a pink solid. *R_f* = 0.31 (n-Heptane:10%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 8.62 (dd, *J* = 8.7, 5.4 Hz, 2H), 7.27 (t, *J* = 8.7 Hz, 2H), 3.10 (s, 3H); ¹³C NMR (101 Hz, CDCl₃) δ 167.2, 165.7 (d, *J* = 254.1 Hz) 163.3, 130.2 (d, *J* = 8.9 Hz),

25

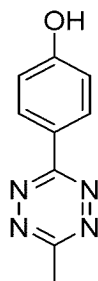
127.9, 116.5 (d, $J=21.9$ Hz), 21.1; HPLC-MS $[M+H]^+$ m/z calc. for $[C_9H_8FN_4]^+$: 191.07; Found: 191.20.

Example 2

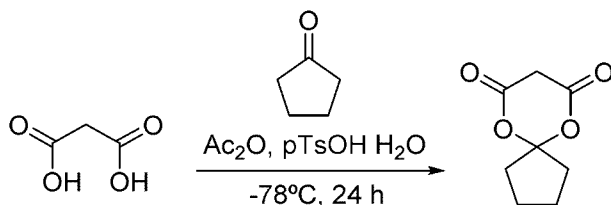


3-(4-iodophenyl)-6-methyl-1,2,4,5-tetrazine (RGV_5): The final compound was obtained from 4-iodobenzonitrile (229 mg, 1.00 mmol) and acetonitrile (261 μ L, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.85 g (39%) of a red solid. $R_f = 0.25$ (n-Heptane:10%EtOAc); 1H NMR (400 MHz, Chloroform- d) δ 8.35 – 8.27 (m, 2H), 8.00 – 7.90 (m, 2H), 3.09 (s, 3H).; ^{13}C NMR (101 MHz, Chloroform- d) δ 167.65, 163.92, 138.71, 131.44, 129.41, 100.33, 21.35.

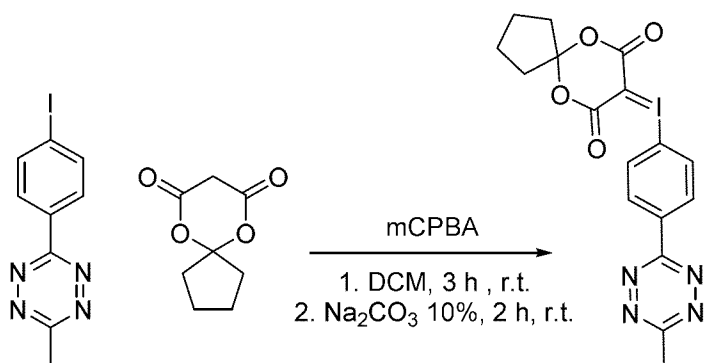
Example 3



4-(1,2,4,5-tetrazin-3-yl)phenol (RGV_59): The final compound was obtained from 4-hydroxybenzonitrile (120 mg, 1.00 mmol) and acetonitrile (261 μ L, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.32 g (20%) of a red solid. $R_f = 0.35$ (n-Heptane:10%EtOAc); 1H NMR (400 MHz, Methanol- d_4) δ 8.42 (d, $J = 8.8$ Hz, 2H), 7.01 (d, $J = 8.8$ Hz, 2H), 3.02 (s, 3H); ^{13}C NMR (101 MHz, MeOD) δ 167.84, 165.19, 163.13, 130.73, 124.33, 117.13, 20.88; HPLC-MS $[M+H]^+$ m/z calc. for $[C_9H_9ON_4]^+$: 189.08; Found: 189.40.

Example 4

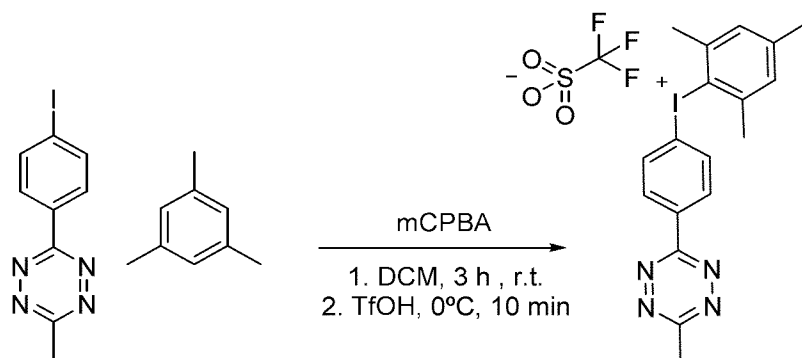
6,10-dioxaspiro[4.5]decane-7,9-dione. Malonic acid (2 g) and pTsOH H₂O were added to a 25 mL flask and cooled to -75 °C. Ac₂O (mL) was added dropwise and then cyclopentanone (mL) under stirring. The reaction was allowed to heat to room temperature overnight. Water was added to the reaction (10 mL) and it was cooled down, until the formation of a white precipitate was observed. The precipitate was then filtered and washed with water and cold EtOH. The white crystal powder was collected to yield 165 mg.

Example 4a**8-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-1,3-iodaneylidene)-6,10-**

dioxaspiro[4.5]decane-7,9-dione (RGV_8). 3-(4-iodophenyl)-6-methyl-1,2,4,5-tetrazine (15 mg, 0.05 mmol, 1 equiv) is dissolved in CH₂Cl₂ (1 mL/1 mmol) in a sealed tube before adding mCPBA (13.8 mg, 0.06 mmol, 1.2 equiv), the mixture is sealed and allowed to stir at room temperature for 3 hours. A solution of 6,10-dioxaspiro[4.5]decane-7,9-dione (9.4 mg, 0.05 mmol, 1.1 equiv) in Na₂CO₃ 10% (2.86 mL/mmole) is prepared and then added dropwise to the mixture in the sealed tube. The mixture was stirred at room temperature for additional 2 hours. To the reaction mixture 5 mL of water is added and is extracted by CH₂Cl₂, dried over MgSO₄, filtered and concentrated *in vacuo*. The crude was submitted to combi flash from 100% CH₂Cl₂ to CH₂Cl₂/10% EtOH. All fractions containing compound were concentrated, dissolved in warm methanol and left to crystallize at 4 °C, which afforded pink crystals (5.4 mg, 15%). R_f = (CH₂Cl₂:EtOH (1:1)); ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.65 (d, *J* = 8.6 Hz, 2H), 8.19 (d, *J* = 8.6 Hz, 2H), 3.11 (s, 3H), 2.16 (t, *J* = 7.4 Hz, 4H), 1.84 (t, *J* = 7.5 Hz, 4H); ¹³C NMR (151

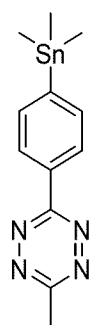
MHz, CDCl₃) δ 168.31, 164.35, 162.97, 138.73, 135.86, 133.86, 131.15, 117.80, 114.58, 37.60, 23.56, 21.46.

Example 5



mesityl(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)iodonium (RGV_10).^[20] In a sealed tube m-Chloroperbenzoic acid (11.3 mg, 0.05 mmol) and 3-(4-iodophenyl)-6-methyl-1,2,4,5-tetrazine (10 mg, 0.03 mmol) were dissolved in CH₂Cl₂ (1 mL/0.23 mmol) and stirred at r.t. during 3 hours. Mesitylene (5.1 μL, 0.04 mmol) is added and the mixture is cooled to 0°C followed by dropwise addition of TfOH (8.9 μL, 0.10 mmol). The reaction mixture was stirred at r.t during 10 minutes. The crude reaction was concentrated under vacuum. Diethyl ether was added and the mixture was stirred at r.t. during 20 minutes and then stored in the freezer during 1 hour for ensure complete precipitation, before filtered and washed with diethyl ether. The resulting solid was collected with methanol and dried under vacuum (12mg, 71%). ¹H NMR (600 MHz, MeOD) δ 8.57 (d, *J* = 8.7 Hz, 2H), 8.07 (d, *J* = 8.7 Hz, 2H), 3.00 (s, 3H), 2.64 (s, 6H), 2.31 (s, 3H).

Example 6



3-(4-trimethyltin)-6-methyl-1,2,4,5-tetrazine (RGV_6). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.46 g (76%)

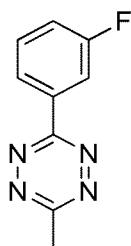
of a pink solid. $R_f = 0.39$ (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 8.52 (d, $J = 8.2$ Hz, 2H), 7.73 (d, $J = 8.2$ Hz, 2H), 3.09 (s, 3H), 0.36 (s, 9H).; ^{13}C NMR (101 MHz, Chloroform- d) δ 167.35, 164.55, 149.30, 136.77, 131.62, 126.81, 21.29, -9.35.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{12}\text{H}_{17}\text{SnN}_4]^+$: 337.04; Found: 337.45.

5

Example 7 – Radiolabeling of Tz-1:

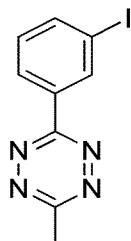
The radiolabelling of Tz-1 was provided as described in Example 128, 129 and 130 and as shown in Figure 2. In the table in Figure 2, the following conditions were applied: [a] $\text{Cu}(\text{OTf})_2$, pyridine, $^{18}\text{F}[\text{KF}]$, DMA, 100 °C, 5 min.; [b] $\text{Cu}(\text{OTf})_2$, pyridine, $^{18}\text{F}[\text{KF}]$ (50 ug K_2CO_3), DMA, 5 min.; [c] $\text{Cu}(\text{OTf})_2$, pyridine, $^{18}\text{F}[\text{KF}]$ (50 ug K_2CO_3), DMA, 100 °C. Radiochemical conversion (RCC) was determined by radio-HPLC ($n=3$). Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step ($n=3$).

15 Example 8



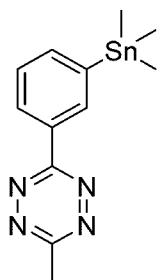
3-(3-fluorophenyl)-6-methyl-1,2,4,5-tetrazine (RGV_49, (W)): The final compound was obtained from 3-fluorobenzonitrile (120 mg, 1.00 mmol) and acetonitrile (261 uL, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.80 g (42%) of a purple solid. $R_f = 0.28$ (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 8.49 – 8.35 (m, 1H), 8.35 – 8.22 (m, 1H), 7.67 – 7.49 (m, 1H), 7.43 – 7.28 (m, 1H), 3.11 (s, 3H); ^{13}C NMR (101 MHz, Chloroform- d) δ 167.79, 163.50 (d, $J = 3.3$ Hz), 163.45 (d, $J = 247.1$ Hz), 134.15 (d, $J = 8.3$ Hz), 131.08 (d, $J = 8.0$ Hz), 123.77 (d, $J = 3.1$ Hz), 119.72 (d, $J = 21.3$ Hz), 114.95 (d, $J = 24.0$ Hz), 21.34; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_9\text{H}_8\text{FN}_4]^+$: 191.07; Found: 191.47.

30

Example 9

3-(3-iodophenyl)-6-methyl-1,2,4,5-tetrazine (RGV_50): The final compound was obtained from 3-iodobenzonitrile (229 mg, 1.00 mmol) and acetonitrile (261 μ L, 5.00 mmol) following the
 5 *General Procedure A*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.88 g (29%) of a purple solid. R_f = 0.26 (n-Heptane:EtOAc=8:1); ^1H NMR (400 MHz, Chloroform- d) δ 8.96 (t, J = 1.7 Hz, 1H), 8.57 (dt, J = 7.9, 1.4 Hz, 1H), 7.96 (ddd, J = 7.9, 1.8, 1.1 Hz, 1H), 7.33 (t, J = 7.9 Hz, 1H), 3.11 (s, 3H); ^{13}C NMR (101 MHz, Chloroform- d) δ 167.81, 163.12, 141.54, 136.84, 133.89, 130.99, 127.15, 94.95, 21.36.

10

Example 9a

3-(3-trimethyltin)-6-methyl-1,2,4,5-tetrazine (RGV_51). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The
 15 crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.32 g (65%) of a pink solid. R_f = 0.38 (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 8.78 – 8.62 (m, 1H), 8.51 (ddd, J = 7.9, 2.0, 1.4 Hz, 1H), 7.74 (dt, J = 7.2, 1.2 Hz, 1H), 7.55 (ddd, J = 7.8, 7.1, 0.6 Hz, 1H), 3.10 (s, 3H), 0.37 (s, 9H); ^{13}C NMR (101 MHz, Chloroform- d) δ 167.31, 164.58, 144.07, 140.15, 135.25, 131.30, 128.78, 127.96, 21.29, -9.26.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{12}\text{H}_{17}\text{SnN}_4]^+$: 337.04; Found: 337.38;

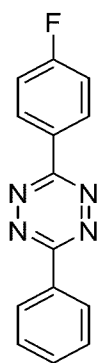
20

Example 10 - Radiolabeling of RGV_49 (W):

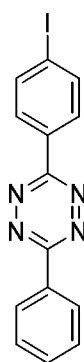
The radiolabelling of RGV_49 was provided as described in Example 128, 129 and 130 and as shown in Table 1 (^{18}F W). In Table 1, the following letters in brackets defines the following:
 25 [a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC ($n=3$); [b] Radiochemical yield (RCY) was decay corrected to the starting amount

of radioactivity received from the cyclotron and the isolated product without formulation step (n=3); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

5

Example 11

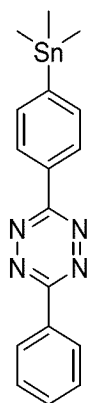
3-(4-fluorophenyl)-6-phenyl-1,2,4,5-tetrazine (RGV_72 (E)): The final compound was obtained from 4-fluorobenzonitrile (121 mg, 1.00 mmol) and benzonitrile (477 μ L, 5.00 mmol) following the *General Procedure A*. The final compound was isolated by preparative TLC (60/30 Toluene/n-Heptane) to yield a pink solid. R_f = 0.65 (Toluene:10%n-Heptane); ^1H NMR (600 MHz, Chloroform- d) δ 8.72 – 8.67 (m, 2H), 8.67 – 8.62 (m, 2H), 7.69 – 7.59 (m, 3H), 7.31 (t, J = 8.6 Hz, 2H); ^{13}C NMR (151 MHz, Chloroform- d) δ 166.83, 164.25 (d, J = 269.7 Hz), 164.11, 132.90, 131.86, 130.46 (d, J = 9.0 Hz), 129.49, 128.19, 128.14, 116.75 (d, J = 22.0 Hz); HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{14}\text{H}_9\text{FN}_4]^+$: 253.09; Found: 253.0.

Example 12

3-(4-iodophenyl)-6-phenyl-1,2,4,5-tetrazine (RGV_14): The final compound was obtained from 4-iodobenzonitrile (229 mg, 1.00 mmol) and benzonitrile (477 μ L, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (90/10 Toluene/n-Heptane) to yield 0.35 g (10%) of a pink solid. R_f = 0.5 (Toluene:10%n-Heptane); ^1H NMR (600 MHz, Chloroform- d) δ 8.68 – 8.63 (m, 2H), 8.40 – 8.35 (m, 2H), 8.00 – 7.95 (m, 2H), 7.68 –

7.59 (m, 3H); ^{13}C NMR (151 MHz, CDCl_3) δ 164.27, 163.80, 138.80, 133.00, 131.80, 131.45, 129.51, 129.43, 128.21, 100.51.

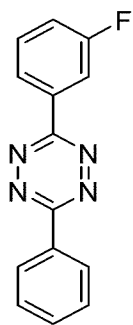
Example 12a



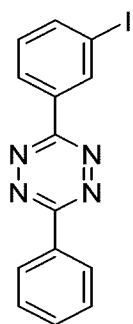
3-phenyl-6-(4-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_26). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.52 mg (95%) of a pink solid. R_f = 0.48 (n-Heptane:10%EtOAc). ^1H NMR (400 MHz, Chloroform- d) δ 8.70 – 8.63 (m, 2H), 8.63 – 8.53 (m, 2H), 7.85 – 7.68 (m, 2H), 7.68 – 7.57 (m, 3H), 0.37 (s, 9H).; ^{13}C NMR (151 MHz, CDCl_3) δ 164.47, 164.13, 149.66, 136.88, 132.80, 132.00, 131.62, 129.46, 128.11, 127.08, -9.32.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{17}\text{H}_{18}\text{N}_4\text{Sn}]^+$: 399.06; Found: 399.1.

Example 13 - Radiolabeling of RGV_72 (E):

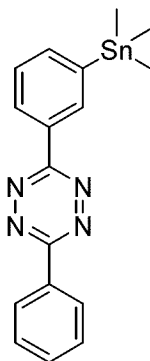
The radiolabelling of RGV_72 was provided as described in Example 128, 129 and 130 and as shown in Table 1 (^{18}F][E). In Table 1, the following letters in brackets defines the following: [a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC (n=3); [b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step (n=3); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

Example 14

3-(3-fluorophenyl)-6-phenyl-1,2,4,5-tetrazine (RGV_71 (R)): The final compound was obtained from 3-fluorobenzonitrile (107 μ L, 1.00 mmol) and benzonitrile (477 μ L, 5.00 mmol) following the *General Procedure A*. The final compound was isolated by preparative TLC (60/30 Toluene/n-Heptane) to yield a pink solid. R_f = 0.65 (Toluene:10%n-Heptane); ^1H NMR (400 MHz, Chloroform- d) δ 8.64 – 8.58 (m, 2H), 8.40 (dt, J = 7.8, 1.3 Hz, 1H), 8.33 – 8.26 (m, 1H), 7.62 – 7.50 (m, 4H), 7.28 (tdd, J = 8.3, 2.7, 1.0 Hz, 1H); ^{13}C NMR (151 MHz, Chloroform- d) δ 164.38, 163.50 (d, J = 247.2 Hz), 163.40 (d, J = 3.1 Hz), 134.17 (d, J = 8.3 Hz), 133.07, 131.76, 131.16 (d, J = 8.0 Hz), 129.53, 128.29, 123.84, 119.84 (d, J = 21.4 Hz), 114.98 (d, J = 24.0 Hz); HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{14}\text{H}_9\text{FN}_4]^+$: 253.09; Found: 253.0..

Example 15

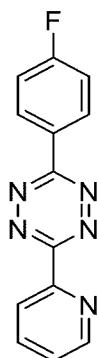
3-(3-iodophenyl)-6-phenyl-1,2,4,5-tetrazine (RGV_15): The final compound was obtained from 3-iodobenzonitrile (229 mg, 1.00 mmol) and benzonitrile (477 μ L, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.50 g (14%) of a pink solid. R_f = 0.5 (Toluene:10%n-Heptane); ^1H NMR (400 MHz, Chloroform- d) δ 9.02 (t, J = 1.8 Hz, 1H), 8.65 (dd, J = 8.0, 1.6 Hz, 2H), 8.62 (d, J = 8.0 Hz, 1H), 7.97 (d, J = 7.9 Hz, 1H), 7.66 – 7.57 (m, 3H), 7.35 (t, J = 7.9 Hz, 1H).; ^{13}C NMR (151 MHz, CDCl_3) δ 164.35, 162.96, 141.60, 136.89, 133.89, 133.07, 131.72, 131.04, 129.52, 128.28, 127.15, 95.03.

Example 16

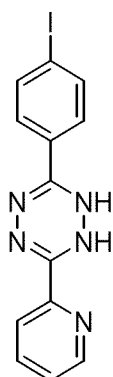
3-phenyl-6-(3-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_25). The final compound
5 was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.53 g (95%) of a pink solid. *R_f* = 0.48 (n-Heptane:10%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 8.78 (dt, *J* = 1.7, 0.7 Hz, 1H), 8.69 – 8.65 (m, 2H), 8.59 (ddd, *J* = 7.9, 2.0, 1.3 Hz, 1H), 7.77 (dt, *J* = 7.2, 1.2 Hz, 1H), 7.65 – 7.61 (m, 3H), 7.58 (ddd, *J* = 7.8, 7.2, 0.6 Hz, 1H), 0.38 (s,
10 9H).; ¹³C NMR (151 MHz, CDCl₃) δ 164.44, 164.06, 144.19, 140.31, 135.34, 132.82, 131.98, 131.28, 129.47, 128.87, 128.10, 128.01, -9.24; HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₇H₁₈N₄Sn]⁺: 399.06; Found: 399.1.

Example 17 - Radiolabeling of RGV_71 (R):

15 The radiolabelling of RGV_71 was provided as described in Example 128, 129 and 130 and as shown in Table 1 ([¹⁸F]R). In Table 1, the following letters in brackets defines the following:
[a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC (n=3); [b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step
20 (n=3); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

Example 18

3-(4-fluorophenyl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (RGV_3, (T)): The final compound was obtained from 4-fluorobenzonitrile (121 mg, 1.00 mmol) and 2-cyanopyridine (520 mg, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (50/50 n-Heptane/EtOAc) to yield 0.40 g (16%) of a pink solid. *R_f* = 0.5 (n-Heptane:50%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 8.92 (ddd, *J* = 4.8, 1.8, 0.9 Hz, 1H), 8.69 – 8.63 (m, 2H), 8.63 (dt, *J* = 7.8, 1.1 Hz, 1H), 7.95 (td, *J* = 7.7, 1.8 Hz, 1H), 7.52 (ddd, *J* = 7.6, 4.8, 1.2 Hz, 1H), 7.28 – 7.19 (m, 2H); ¹³C NMR (151 MHz, Chloroform-*d*) δ 167.07, 164.57 (d, *J* = 243.4 Hz), 163.51, 151.03, 150.32, 137.69, 130.97 (d, *J* = 9.2 Hz), 127.93 (d, *J* = 3.0 Hz), 126.55, 124.08, 116.82 (d, *J* = 22.1 Hz); HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₁₃H₈FN₅]⁺: 254.08; Found: 254.0.

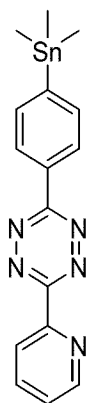
Example 19

3-(4-iodophenyl)-6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazine (RGV_16): The final compound was obtained from 4-iodobenzonitrile (229 mg, 1.00 mmol) and 2-cyanopyridine (433 μL, 4.5 mmol) following the *General Procedure B*. The crude was purified using flash chromatography (90/10 Toluene/EtOAc) to yield 0.71 g (19%) of an orange solid. *R_f* = 0.38 (Toluene/10%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.31 (s, 1H), 8.74 (s, 1H), 8.63 (d, *J* = 4.9 Hz, 1H), 8.00 – 7.86 (m, 2H), 7.85 – 7.75 (m, 2H), 7.61 (d, *J* = 8.4 Hz, 2H),

7.53 (ddd, $J = 6.9, 4.8, 1.6$ Hz, 1H).; ^{13}C NMR (151 MHz, CDCl_3) δ 148.43, 147.14, 147.10, 138.15, 137.15, 129.79, 127.59, 125.29, 121.53, 97.03.

Example 20

5

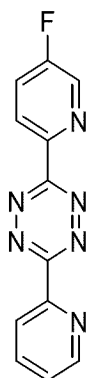


3-(pyridin-2-yl)-6-(4-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_46). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The final compound autooxidised under reaction conditions. The crude was purified using flash chromatography (60/40 n-Heptane/EtOAc) to yield 14 mg (49%) of a pink solid.. $R_f = 0.31$ (EtOAc:50%n-Heptane); ^1H NMR (600 MHz, Chloroform- d) δ 9.00 – 8.94 (m, 1H), 8.69 (dt, $J = 7.9, 1.0$ Hz, 1H), 8.64 – 8.60 (m, 2H), 8.00 (td, $J = 7.8, 1.8$ Hz, 1H), 7.78 – 7.74 (m, 2H), 7.59 – 7.52 (m, 1H), 0.37 (s, 9H); ^{13}C NMR (151 MHz, CDCl_3) δ 164.90, 163.61, 151.07, 150.53, 150.25, 137.57, 136.90, 131.37, 127.48, 126.43, 124.02, -9.33.; HPLC-MS [M+H] $^+$ m/z calc. for $[\text{C}_{16}\text{H}_{17}\text{N}_5\text{Sn}]^+$: 400.06; Found: 400.1.

Example 21 - Radiolabeling of RGV_3 (T):

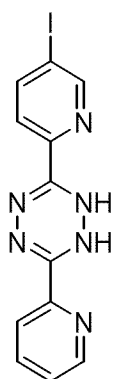
The radiolabelling of RGV_3 was provided as described in Example 128, 129 and 130 and as shown in Table 1 (^{18}F T). In Table 1, the following letters in brackets defines the following: [a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC (n=3); [b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step (n=3); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

Example 22

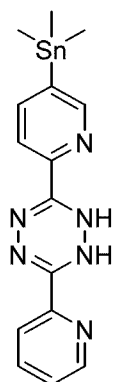


3-(5-fluoropyridin-2-yl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (RGV_61, (U)): The final compound was obtained from 5-fluoropicolinonitrile (122 mg, 1.00 mmol) and 2-cyanopyridine (520 μ L, 5.00 mmol) following the *General Procedure A*. The crude was purified using flash chromatography (50/50 n-Heptane/EtOAc) to yield 0.45 g (18%) of a pink solid. R_f = 0.5 (n-Heptane:50%EtOAc); HPLC-MS $[M+H]^+$ m/z calc. for $[C_{12}H_8FN_6]^+$: 255.08; Found: 255.0.

Example 23

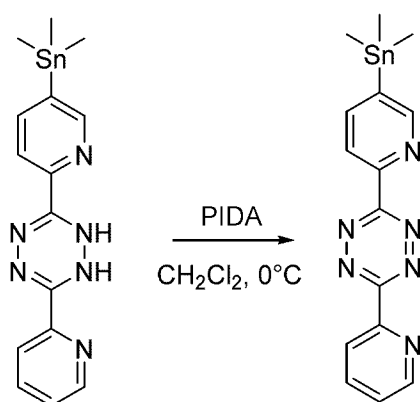


3-(5-iodopyridin-2-yl)-6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazine (CPO_5):^[12] The final compound was obtained from 2-Cyano-5-iodopyridine (231 mg, 1.00 mmol) and 2-Cyanopyridine (433 μ L, 4.5 mmol) following the *General Procedure B*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield 0.53 g (15%) of an orange solid. R_f = 0.44 (n-Heptane:5%EtOAc); 1H NMR (600 MHz, Chloroform- d) δ 8.79 (dd, J = 2.1, 0.8 Hz, 1H), 8.61 – 8.54 (m, 2H), 8.40 (s, 1H), 8.08 – 8.02 (m, 2H), 7.83 (dd, J = 8.3, 0.9 Hz, 1H), 7.75 (td, J = 7.7, 1.7 Hz, 1H), 7.35 (ddd, J = 7.5, 4.9, 1.2 Hz, 1H).; ^{13}C NMR (151 MHz, $CDCl_3$) δ 154.62, 148.54, 147.50, 146.66, 146.55, 146.21, 145.15, 136.89, 125.09, 122.91, 121.46, 95.02.

Example 24**3-(pyridin-2-yl)-6-(5-(trimethylstannyl)pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazine**

(CPO₆). The final compound was obtained from 50 mg (0.17 mmol) of the starting material,

following the *General Procedure D.1*. The crude was purified using flash chromatography (70/30 n-Heptane/EtOAc) to yield 0.47 g (85%) of an orange solid. *R_f* = 0.50 (n-Heptane:10%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 8.62 – 8.51 (m, 4H), 8.05 (dt, *J* = 8.0, 1.1 Hz, 1H), 7.97 (dd, *J* = 7.7, 1.1 Hz, 1H), 7.84 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.75 (td, *J* = 7.7, 1.7 Hz, 1H), 7.34 (ddd, *J* = 7.5, 4.9, 1.2 Hz, 1H), 0.36 (s, 9H).; ¹³C NMR (101 MHz, CDCl₃) δ 154.24, 148.52, 147.74, 147.18, 147.10, 146.81, 144.22, 139.76, 136.82, 124.95, 121.41, 121.02, -9.35.

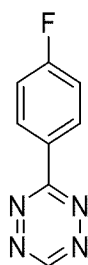
Example 25

3-(pyridin-2-yl)-6-(5-(trimethylstannyl)pyridin-2-yl)-1,2,4,5-tetrazine (RGV₆₀). The 1,2-dihydro-1,2,4,5-tetrazine stannate was dissolved in dry CH₂Cl₂ and cooled to 0°C, followed by the portion wise addition of (Diacetoxyiodo)benzene (1.2 equiv.). The reaction was allowed to warm to r.t. and stirred for 3h. The crude was purified using flash chromatography. HPLC-MS [M+H]⁺ m/z calc. for [C₁₅H₁₆N₆Sn]⁺: 401.05; Found: 401.1..

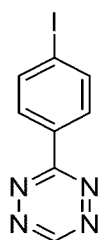
Example 26 - Radiolabeling of RGV_61 (U):

The radiolabelling of RGV_61 was provided as described in Example 128, 129 and 130 and as shown in Table 1 ($[^{18}\text{F}]\text{U}$). In Table 1, the following letters in brackets defines the following:

[a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC (n=3); [b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step (n=3); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

Example 27

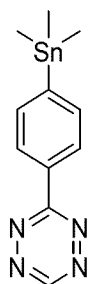
3-(4-fluorophenyl)-1,2,4,5-tetrazine (RGV_55, (Y)): The final compound was obtained from 4-fluorobenzonitrile (242 mg, 4 mmol) following *General Procedure C*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.12 g (34%) of a red solid. R_f = 0.33 (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.21 (s, 1H), 8.70 – 8.61 (m, 2H), 7.29 (t, J = 8.7 Hz, 2H); ^{13}C NMR (101 MHz, Chloroform- d) δ 167.52 (d, J = 255.2 Hz), 164.98, 161.85 (d, J = 798.2 Hz), 130.85 (d, J = 9.2 Hz), 127.94 (d, J = 3.2 Hz), 116.81 (d, J = 22.1 Hz); HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_8\text{H}_6\text{FN}_4]^+$: 177.06; Found: 177.34..

Example 28

3-(4-iodophenyl)-1,2,4,5-tetrazine (RGV_56): The final compound was obtained from 4-iodobenzonitrile (458 mg, 4 mmol) following *General Procedure C*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.16 mg (27%) of a pink solid. R_f = 0.37 (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.24 (s, 1H), 8.39 –

8.32 (m, 2H), 8.02 – 7.95 (m, 2H).; ^{13}C NMR (101 MHz, CDCl_3) δ 166.36, 158.10, 138.90, 131.24, 129.75, 101.22.

Example 29

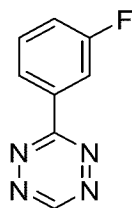


3-(4-trimethyltin)-6-methyl-1,2,4,5-tetrazine (RGV_57). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.27 g (61%) of a pink solid. R_f = 0.43 (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.20 (s, 1H), 8.58 – 8.54 (m, 2H), 7.84 – 7.67 (m, 2H), 0.37 (s, 9H).; ^{13}C NMR (101 MHz, Chloroform- d) δ 167.00, 157.96, 150.34, 136.90, 130.29, 127.35, -9.33.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{11}\text{H}_{15}\text{SnN}_4]^+$: 323.04; Found: 323.38.

Example 30 - Radiolabeling of RGV_55 (Y):

The radiolabelling of RGV_55 was provided as described in Example 128, 129 and 130 and as shown in Table 1. In Table 1 ($[\text{F}^{18}]\text{Y}$), the following letters in brackets defines the following: [a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC ($n=3$); [b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step ($n=3$); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

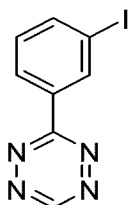
Example 31



3-(3-fluorophenyl)-1,2,4,5-tetrazine (RGV-52.(9)). The final compound was obtained from 3-fluorobenzonitrile (242 mg, 4 mmol) following *General Procedure C*. The crude was purified

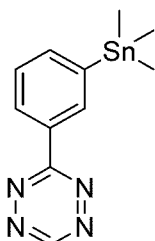
using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.12 g (34%) of a red solid. R_f = 0.34 (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.25 (s, 1H), 8.44 (dt, J = 7.8, 1.3 Hz, 1H), 8.33 (ddd, J = 9.7, 2.7, 1.6 Hz, 1H), 7.60 (td, J = 8.1, 5.7 Hz, 1H), 7.36 (tdd, J = 8.3, 2.7, 1.0 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform- d) δ 165.87 (d, J = 3.3 Hz), 163.47 (d, J = 247.6 Hz), 158.17, 133.91 (d, J = 8.2 Hz), 131.23 (d, J = 8.0 Hz), 124.18 (d, J = 3.2 Hz), 120.35 (d, J = 21.3 Hz), 115.32 (d, J = 24.1 Hz); HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_8\text{H}_6\text{FN}_4]^+$: 177.05; Found: 177.54.

Example 32



3-(3-iodophenyl)-1,2,4,5-tetrazine (RGV_53): The final compound was obtained from 3-iodobenzonitrile (458 mg, 4 mmol) following *General Procedure C*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.19 g (33%) of a pink solid. R_f = 0.36 (n-Heptane:10%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.25 (s, 1H), 9.00 (t, J = 1.7 Hz, 1H), 8.60 (ddd, J = 7.9, 1.7, 1.1 Hz, 1H), 7.99 (ddd, J = 7.9, 1.8, 1.0 Hz, 1H), 7.35 (t, J = 7.9 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3) δ 165.47, 158.17, 142.11, 137.21, 133.63, 131.08, 127.51, 95.02.

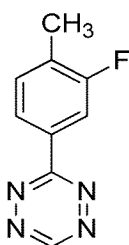
Example 33



3-(3-trimethyltin)-1,2,4,5-tetrazine (RGV_54). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.28 g (58%) of a pink solid. R_f = 0.30 (n-Heptane:10%EtOAc); ^1H NMR (400MHz, Chloroform- d) δ 10.14 (s, 1H), 8.67 (s, 1H), 8.48 (s, J =7.9 Hz, 1H) 7.72 (d, J =7.5 Hz, 1H) 7.50 (t, J =7.9, 15.8 Hz, 1H) 0.30 (s, 9H); ^{13}C NMR (101 MHz, Chloroform- d) δ 166.83, 157.75, 144.16, 140.60, 135.48, 130.96, 128.74 128.17, -9.38.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{11}\text{H}_{15}\text{SnN}_4]^+$: 323.04; Found: 323.38.

Example 34 - Radiolabeling of RGV_52 (9):

The radiolabelling of RGV_49 was provided as described Example 128, 129 and 130 and as shown in Table 1 ($[^{18}\text{F}]9$). In Table 1, the following letters in brackets defines the following: [a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC (n=3); [b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step (n=3); [c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in CAN; [d] No product formed or could not be isolated.

Example 35

3-(3-fluoro-4-methylphenyl)-1,2,4,5-tetrazine (UB-007). The final compound was obtained from 3-fluoro-4-methylbenzonitrile (0.54 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield after crystallization with n-Heptane 0.21 g (28%) of a red solid. $R_f = 0.4$ (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.21 (s, 1H), 8.33 (dd, $J = 8.0, 1.7$ Hz, 1H), 8.27 (dd, $J = 10.5, 1.7$ Hz, 1H), 7.43 (t, $J = 7.8$ Hz, 1H), 2.41 (s, 3H); ^{13}C NMR (101 MHz, Chloroform- d) δ 165.80, 161.82 (d, $J = 246.1$ Hz), 157.83, 132.50 (d, $J = 8.3$ Hz), 131.11 (d, $J = 8.3$ Hz), 130.94 (d, $J = 17.4$ Hz), 123.78 (d, $J = 3.5$ Hz), 114.69 (d, $J = 25.1$ Hz), ; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_9\text{H}_8\text{FN}_4]^+$: 191.07; found 191.11 .

Example 36 Radiolabeling of UB-007:

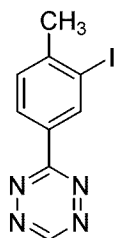
The radiolabelling of UB007 was provided as described in Example 128, 129 and 130 and as shown in Table 2 annexed to this description. In Table 2, the following letters in brackets defines the following:

[a] Stannate precursor could not be synthesized.

[b] No tetrazine formation detected.

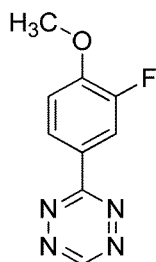
[c] Decomposition during Cu-mediated ^{18}F -fluorination reaction.

[d] RCCs were determined by radio-HPLC (n = 3).



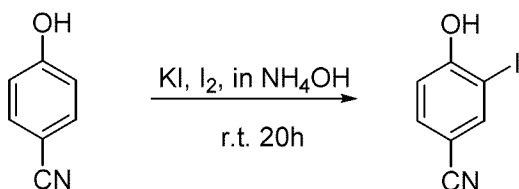
3-(3-iodo-4-methylphenyl)-1,2,4,5-tetrazine (RGV_100). The final compound was obtained from 3-iodo-4-methylbenzonitrile (972 mg, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) followed by
 5 recrystallization from n-Heptane afforded 0.21 g (18%) of a red solid. *R_f* = 0.42 (n-Heptane:10%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.21 (s, 1H), 9.08 (s, 1H), 8.50 (d, *J* = 9.7 Hz, 1H), 7.46 (d, *J* = 8.0 Hz, 1H), 2.56 (s, 3H); ¹³C NMR (151 MHz, CDCl₃) δ 165.24, 157.88, 147.21, 138.55, 130.72, 130.45, 127.85, 101.69, 28.46.

10 Example 37



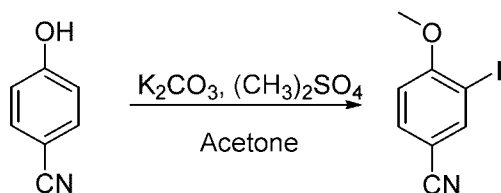
3-(3-fluoro-4-methoxyphenyl)-1,2,4,5-tetrazine (UB-008). The final compound was obtained from 3-Fluoro-4-methoxybenzonitrile (0.60 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) followed by
 15 recrystallization from n-Heptane afforded 0.24 g (29%) of a red solid. *R_f* = 0.39 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.18 (s, 1H), 8.45 (ddd, *J* = 8.6, 2.1, 1.3 Hz, 1H), 8.38 (dd, *J* = 12.1, 2.2 Hz, 1H), 7.18 (t, *J* = 8.5 Hz, 1H), 4.04 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 165.53, 157.51, 152.72 (d, *J* = 247.7 Hz), 152.06 (d, *J* = 10.7 Hz), 125.29 (d, *J* = 3.6 Hz), 124.35 (d, *J* = 7.2 Hz), 115.80 (d, *J* = 20.8 Hz), 113.52 (d, *J* = 2.2 Hz),
 20 56.36; HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₉H₈FN₄O]⁺: 207.06; found 207.08

Example 38

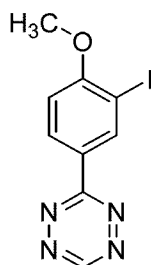


4-hydroxy-3-iodobenzonitrile. The preparation of this intermediates, was performed using a method described previously.^[16] To a solution of 4-hydroxybenzonitrile (4 g, 33.6 mmol) in 25% NH₄OH (180 mL) was added a mixture of KI (27.31 g, 167.9 mmol), I₂ (9.38 g, 36.9 mmol) in H₂O (40 mL). The reaction was allowed to stir at r.t. for 20 hours, in which time the mixture colour gradually turned from black to a white thick suspension. The precipitate formed was filtered off and the filtrate concentrated. The residue was then dissolved in CH₂Cl₂ and washed with H₂O, saturated aqueous Na₂S₂O₃ solution, and brine. The organic layer was dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield the product 6.52 g (79%). ¹H NMR (400 Hz, Chloroform-*d*) δ 7.97 (d, *J* = 1.9 Hz, 1H), 7.55 (dd, *J* = 8.5, 1.95 Hz, 1H), 7.05 (d, *J* = 8.5 Hz, 1H), 5.87 (s, 1H); ¹³C NMR (600 Hz, CDCl₃) δ 158.72, 142.17, 134.28, 117.30, 116.31, 106.02, 85.48.

Example 39

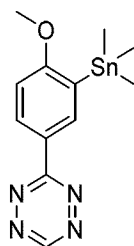


3-iodo-4-methoxybenzonitrile. The preparation of this intermediate, was performed using a method described previously.^[17] 4-Hydroxy-3-iodobenzonitrile (6.52 g, 26.6 mmol) and K₂CO₃ (11.03 g, 79.8 mmol) were suspended in acetone (130 mL) before (CH₃)₂SO₄ (5.03 g, 79.8 mmol) was added. Then the flask is fitted with a reflux condenser and the mixture was heated to 70°C for 90 minutes after which the mixture became a pale yellow thick suspension. After cooling to room temperature, the mixture was filtered, and the filter cake was washed with additional acetone. The solvent was removed on a rotary evaporator and the residue was suspended in water (250 mL) for 90 minutes. The precipitate was filtered off and dried overnight. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield the product 6.06 g (88%). ¹H NMR (400 Hz, Chloroform-*d*) δ 8.05 (d, *J* = 2.0 Hz, 1H), 7.64 (dd, *J* = 8.6, 2.0 Hz, 1H), 6.85 (d, *J* = 8.6 Hz, 1H), 3.95 (s, 3H); ¹³C NMR (600 Hz, CDCl₃) δ 161.56, 142.81, 134.08, 117.55, 110.71, 105.94, 86.04, 56.72.

Example 40

3-(3-iodo-4-methoxyphenyl)-1,2,4,5-tetrazine (RGV_106): The final compound was obtained from 3-iodo-4-methoxybenzonitrile (1.03 g, 4.00 mmol) following *General Procedure*
 5 C. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield after recrystallization with n-Heptane 0.26 g (20%) as a red solid. *R_f* = 0.30 (n-Heptane:20%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.16 (s, 1H), 9.07 (s, 1H), 8.62 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.01 (d, *J* = 8.7 Hz, 1H), 4.01 (s, 3H); ¹³C NMR (151 MHz, CDCl₃) δ 165.20, 162.20, 157.70, 139.67, 130.28, 125.84, 111.17, 86.92, 56.85.

10

Example 41

3-(4-methoxy-3-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_109). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General*
 15 *Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.20 g (36%) of a pink solid. *R_f* = 0.28 (n-Heptane:10%EtOAc); ¹H NMR (400Hz, Chloroform-*d*) δ 8.67-8.65 (m, 2H), δ 8.59 (d, *J* = 8.1 Hz, 2H), δ 7.76 (d, *J* = 8.1 Hz, 2H), δ 8.67-8.65 (m, 3H), δ 0.37 (s, 9H); ¹³C NMR (151 MHz, Chloroform-*d*) δ

Example 42 of UB-008:

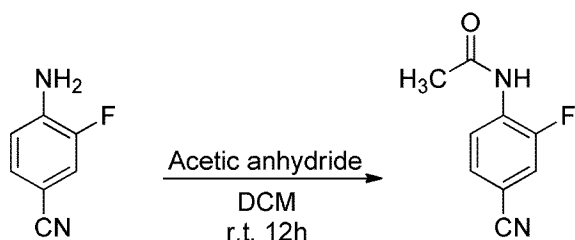
The radiolabelling of UB008 was provided as described in Example 128, 129 and 130 and as shown in Table 2. In Table 2, the following letters in brackets defines the following:

[a] Stannate precursor could not be synthesized.

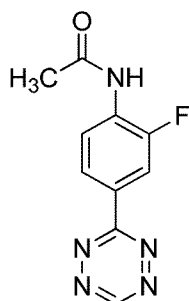
[b] No tetrazine formation detected.

25 [c] Decomposition during Cu-mediated ¹⁸F-fluorination reaction.

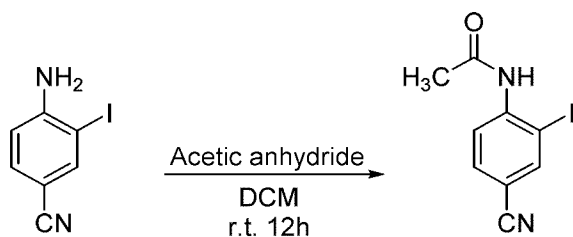
[d] RCCs were determined by radio-HPLC (n = 3).

Example 43

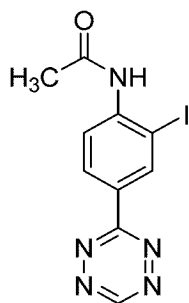
N-(4-cyano-2-fluorophenyl)acetamide: The preparation of this intermediate, was performed using a method described previously.^[18] To a solution of 4-amino-3-fluorobenzonitrile (0.82 g, 6.00 mmol) in CH₂Cl₂ (30.0 mL) was added acetic anhydride (0.80 mL, 8.40 mmol). The mixture was stirred at room temperature for 12 hours. The suspension was filtered, and the solvent removed under reduced pressure. Purification by flash chromatography (70/30 n-Heptane/EtOAc) afforded 0.90 g of N-(4-cyano-2-fluorophenyl)acetamide as a white solid. *R_f* = 0.27 (n-Heptane:40%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.12 (s, 1H), 8.28 (t, *J* = 8.2 Hz, 1H), 7.88 (dd, *J* = 11.1, 1.9 Hz, 1H), 7.65 (dt, *J* = 8.5, 1.3 Hz, 1H), 2.15 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 169.9, 152.05 (d, *J* = 247.2 Hz), 132.10 (d, *J* = 11.2 Hz), 129.82 (d, *J* = 3.6 Hz), 123.31 (d, *J* = 2.9 Hz), 119.77 (d, *J* = 23.4 Hz), 118.36 (d, *J* = 2.7 Hz), 106.18 (d, *J* = 9.4 Hz), 24.3.

Example 44

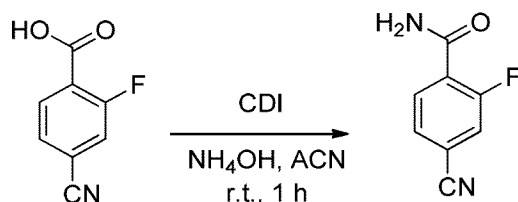
N-(2-Fluoro-4-(1,2,4,5-tetrazin-3-yl)phenyl)acetamide (UB-148): The final compound was obtained from N-(4-cyano-2-fluorophenyl)acetamide (0.71 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (60/40 n-Heptane/EtOAc) to yield 0.37 g (40%) of UB-148 as a red solid. *R_f* = 0.25 (n-Heptane:40%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.58 (s, 1H), 10.09 (s, 1H), 8.45 – 8.21 (m, 3H), 2.18 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 169.79, 164.91 (d, *J* = 3.0 Hz), 158.44, 153.21 (d, *J* = 246.0 Hz), 131.38 (d, *J* = 11.3 Hz), 128.01 (d, *J* = 7.9 Hz), 124.72 (d, *J* = 3.3 Hz), 123.67, 114.75 (d, *J* = 22.1 Hz), 24.3; HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₁₀H₉FN₅O]⁺: 234.08; found 234.10

Example 45

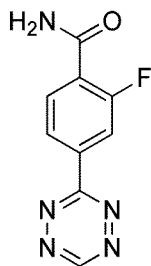
N-(4-cyano-2-iodophenyl)acetamide (RGV_136): The preparation of this intermediate, was performed using a method described previously.^[18] To a solution of the corresponding aniline (1.5 g, 6.00 mmol) in CH₂Cl₂ (30.0 mL) was added acetic anhydride (0.85 mL, 9 mmol). The mixture was stirred at room temperature for 12 hours. The suspension was filtered, and the solvent removed under vacuum. Purification by flash chromatography (70/30 n-Heptane/EtOAc) afforded 0.90 g (52%) of RGV_136 as a white solid. *R_f* = 0.5 (n-Heptane:40%EtOAc); ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.50 (s, 1H), 8.36 (d, *J* = 1.9 Hz, 1H), 7.82 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 2.12 (s, 3H).; ¹³C NMR (151 MHz, DMSO) δ 168.72, 144.08, 142.44, 132.41, 125.76, 117.29, 108.88, 94.41, 23.51.

Example 46

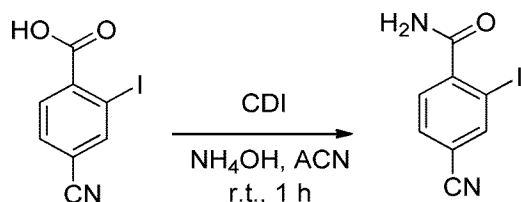
N-(2-iodo-4-(1,2,4,5-tetrazin-3-yl)phenyl)acetamide (RGV_144): The final compound was obtained from N-(4-cyano-2-iodophenyl)acetamide (1.14 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (60/40 n-Heptane/EtOAc) to yield 0.29 g (21%) of RGV_144 as a pink solid. *R_f* = 0.29 (n-Heptane:40%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.20 (s, 1H), 9.07 (s, 1H), 8.62 (d, *J* = 8.7 Hz, 1H), 8.58 (d, *J* = 8.6 Hz, 1H), 7.71 (s, 1H), 2.32 (s, 2H).; ¹³C NMR (151 MHz, CDCl₃) δ 168.57, 164.98, 157.88, 142.56, 138.78, 129.60, 128.51, 121.17, 25.27, 1.16.

Example 47

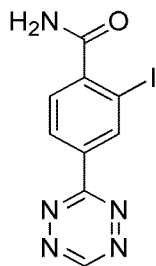
- 4-Cyano-2-fluorobenzamide (UB-20).** To a solution of 4-cyano-2-fluorobenzoic acid (0.99 g, 6.0 mmol) in acetonitrile (20 ml) was added 1,1'-carbonyldiimidazole (1.46 g, 9.0 mmol). The mixture was stirred at room temperature for 45 minutes, before addition of aqueous ammonium hydroxide solution (35%, 20 ml). The reaction mixture was stirred for 45 minutes and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the 0.78 g (79%) of UB-20 as a white solid. *R_f* = 0.25 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.02 – 7.91 (m, 2H), 7.86 (s, 1H), 7.80 – 7.74 (m, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 164.56, 158.89 (d, *J* = 251.4 Hz), 131.56 (d, *J* = 4.0 Hz), 129.60 (d, *J* = 15.7 Hz), 129.16 (d, *J* = 4.0 Hz), 120.79 (d, *J* = 26.7 Hz), 117.68 (d, *J* = 2.8 Hz), 114.58 (d, *J* = 10.0 Hz).

Example 48

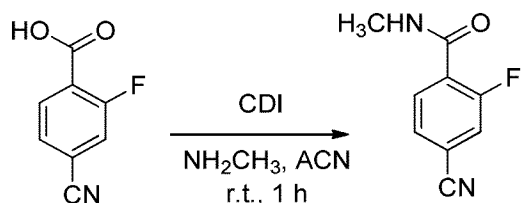
- 2-Fluoro-4-(1,2,4,5-tetrazin-3-yl)benzamide (UB-22).** The final compound was obtained from 4-cyano-2-fluorobenzamide (0.78 g, 4.75 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 CH₂Cl₂/MeOH) to yield 0.21 g (20%) of UB22 as a red solid. *R_f* = 0.30 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.68 (s, 1H), 8.39 (dd, *J* = 8.0, 1.6 Hz, 1H), 8.30 (dd, *J* = 11.1, 1.6 Hz, 1H), 7.97 (s, 1H), 7.93 (t, *J* = 7.7 Hz, 1H), 7.83 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.07, 164.84 (d, *J* = 2.9 Hz), 159.91 (d, *J* = 250.1 Hz), 158.83, 136.15 (d, *J* = 8.5 Hz), 131.81 (d, *J* = 3.4 Hz), 128.25 (d, *J* = 15.2 Hz), 124.16 (d, *J* = 3.4 Hz), 115.57 (d, *J* = 25.4 Hz); HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₉H₇FN₅O]⁺: 220.06; found 226.07.

Example 49

4-Cyano-2-iodobenzamide (UB-282). To a solution of 4-cyano-2-iodobenzoic acid (1.00 g, 3.66 mmol) in acetonitrile (20 ml) was added 1,1'-carbonyldiimidazole (0.89 g, 5.49 mmol). The mixture was stirred at room temperature for 45 minutes, before addition of aqueous ammonium hydroxide solution (35%, 20 ml). The reaction mixture was stirred for 45 minutes and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the 0.89 g (89%) of UB-282 as a white solid. $R_f = 0.23$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 8.37 (d, $J = 1.5$ Hz, 1H), 7.98 (s, 1H), 7.91 (dd, $J = 7.9, 1.6$ Hz, 1H), 7.72 (s, 1H), 7.48 (d, $J = 7.8$ Hz, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 170.11, 148.12, 142.53, 132.32, 128.42, 117.40, 113.37, 94.10.

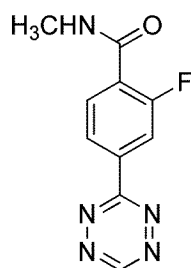
Example 50

2-Iodo-4-(1,2,4,5-tetrazin-3-yl)benzamide (UB-283). The final compound was obtained from 4-cyano-2-iodobenzamide (0.50 g, 1.83 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) to yield 0.25 g (41%) of UB-283 as a red solid. $R_f = 0.32$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 10.66 (s, 1H), 8.89 (d, $J = 1.6$ Hz, 1H), 8.52 (dd, $J = 8.0, 1.7$ Hz, 1H), 8.02 (s, 1H), 7.71 (s, 1H), 7.63 (d, $J = 8.0$ Hz, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 170.57, 164.55, 158.77, 147.21, 138.45, 134.16, 128.99, 127.70, 94.43.

Example 51

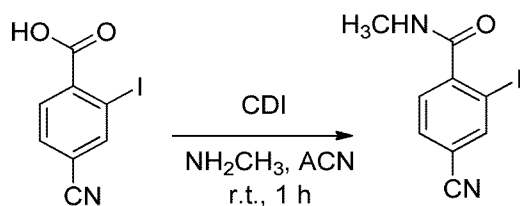
4-Cyano-2-fluoro-N-methylbenzamide (UB-198). To a solution of 4-cyano-2-fluorobenzoic acid (0.99 g, 6.0 mmol) in acetonitrile (20 ml) was added 1,1'-carbonyldiimidazole (1.46 g, 9.0 mmol). The mixture was stirred at room temperature for 45 minutes, before addition of aqueous methylamine solution (40%, 20 ml). The reaction mixture was stirred for 45 minutes and ice cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the 0.86 g (80%) of UB-198 as a white solid. $R_f = 0.29$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 8.50 (d, $J = 6.3$ Hz, 1H), 8.02 – 7.92 (m, 1H), 7.81 – 7.71 (m, 2H), 2.79 (d, $J = 4.6$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 163.25, 158.82 (d, $J = 251.2$ Hz), 131.56 (d, $J = 3.8$ Hz), 129.54 (d, $J = 15.6$ Hz), 129.23 (d, $J = 3.9$ Hz), 120.78 (d, $J = 26.6$ Hz), 117.67 (d, $J = 2.9$ Hz), 114.55 (d, $J = 10.1$ Hz), 26.69.

Example 52



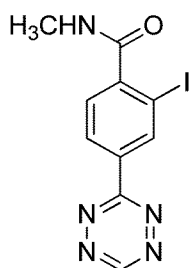
2-Fluoro-N-methyl-4-(1,2,4,5-tetrazin-3-yl)benzamide (UB-204). The final compound was obtained from 4-cyano-2-fluoro-N-methylbenzamide (0.77 g, 4.32 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) to yield 0.36 g (36%) of UB-204 as a red solid. $R_f = 0.35$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 10.68 (s, 1H), 8.50 (d, $J = 5.3$ Hz, 1H), 8.40 (dd, $J = 8.1, 1.6$ Hz, 1H), 8.30 (dd, $J = 11.1, 1.6$ Hz, 1H), 7.91 (t, $J = 7.7$ Hz, 1H), 2.83 (d, $J = 4.6$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 164.83 (d, $J = 2.9$ Hz), 163.79, 159.81 (d, $J = 249.8$ Hz), 158.82, 136.08 (d, $J = 8.5$ Hz), 131.76 (d, $J = 3.5$ Hz), 128.25 (d, $J = 15.4$ Hz), 124.23 (d, $J = 3.3$ Hz), 115.57 (d, $J = 25.4$ Hz), 26.75; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{10}\text{H}_9\text{FN}_5\text{O}]^+$: 234.08; found 234.10.

Example 53



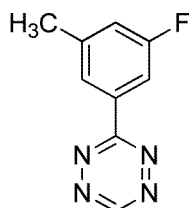
4-Cyano-2-iodo-N-methylbenzamide (UB-306). To a solution of 4-cyano-2-iodobenzoic acid (1.00 g, 3.66 mmol) in acetonitrile (20 ml) was added 1,1'-carbonyldiimidazole (0.89 g, 5.49 mmol). The mixture was stirred at room temperature for 45 minutes, before addition of aqueous methylamine solution (40%, 20 ml). The reaction mixture was stirred for 45 minutes and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the 0.85 g (81%) of UB-306 as a white solid. $R_f = 0.31$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 8.44 (d, $J = 4.8$ Hz, 1H), 8.38 (d, $J = 1.5$ Hz, 1H), 7.91 (dd, $J = 7.8, 1.6$ Hz, 1H), 7.47 (d, $J = 7.8$ Hz, 1H), 2.76 (d, $J = 4.6$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 168.77, 148.09, 142.44, 132.36, 128.67, 117.37, 113.50, 94.63, 26.41.

Example 54



2-Iodo-N-methyl-4-(1,2,4,5-tetrazin-3-yl)benzamide (UB-318). The final compound was obtained from 4-cyano-2-iodo-N-methylbenzamide (0.60 g, 2.09 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 CH₂Cl₂/MeOH) to yield 0.25 g (35%) of UB-318 as a red solid. $R_f = 0.20$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 10.66 (s, 1H), 8.89 (d, $J = 1.6$ Hz, 1H), 8.57 – 8.44 (m, 2H), 7.60 (d, $J = 8.0$ Hz, 1H), 2.81 (d, $J = 4.6$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 169.20, 164.54, 158.77, 147.24, 138.33, 134.27, 129.20, 127.73, 94.92, 26.47.

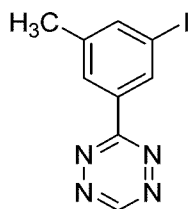
Example 55



3-(3-fluoro-5-methylphenyl)-1,2,4,5-tetrazine (UB-052). The final compound was obtained from 3-Fluoro-5-methylbenzonitrile (0.54 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield 0.26 g (34%)

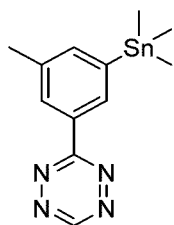
of a red oil. $R_f = 0.39$ (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.16 (s, 1H), 8.19 (d, $J = 1.4$ Hz, 1H), 8.05 (d, $J = 9.4$ Hz, 1H), 7.10 (d, $J = 9.2$ Hz, 1H), 2.43 (s, 3H); ^{13}C NMR (101 MHz, Chloroform- d) δ 165.83 (d, $J = 3.4$ Hz), 163.30 (d, $J = 246.8$ Hz), 157.95, 141.81 (d, $J = 7.7$ Hz), 133.31 (d, $J = 8.9$ Hz), 124.67 (d, $J = 2.7$ Hz), 120.79 (d, $J = 21.2$ Hz), 112.29 (d, $J = 24.3$ Hz), 21.45 (d, $J = 1.8$ Hz); HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_9\text{H}_8\text{FN}_4]^+$: 191.07; found 191.08.

Example 56



- 10 **3-(3-iodo-5-methylphenyl)-1,2,4,5-tetrazine (RGV_114).** The final compound was obtained from 3-iodo-5-methylbenzonitrile (0.97 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield after recrystallization with n-Heptane 0.27 g (22%) as a red solid. $R_f = 0.45$ (n-Heptane:20%EtOAc) ^1H NMR (400 MHz, Chloroform- d) δ 10.22 (s, 1H), 8.83 – 8.67 (m, 1H), 8.44 – 8.28 (m, 1H), 7.87 – 7.74 (m, 1H), 2.43 (s, 2H); ^{13}C NMR (151 MHz, CDCl_3) δ 165.49, 158.09, 142.71, 141.41, 134.34, 133.30, 128.22, 94.99, 21.21.

Example 57



- 20 **3-(3-methyl-5-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_123).** The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure D.2*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.25 g (27%) of a pink solid. $R_f = 0.34$ (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.20 (s, 1H), 8.65 – 8.50 (m, 1H), 8.48 – 8.33 (m, 1H), 7.73 – 7.43 (m, 1H), 2.48 (s, 3H), 0.36 (s, 9H); ^{13}C NMR (151 MHz, CDCl_3) δ 167.05, 157.86, 144.08, 141.58, 138.56, 132.79, 131.00, 128.91, 21.58, -9.25.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{12}\text{H}_{16}\text{N}_4\text{Sn}]^+$: 337.05; Found: 337.1.

Example 58 of UB-052:

The radiolabelling of UB-052 was provided as described in Example 128, 129 and 130 and as shown in Table 2. In Table 2, the following letters in brackets defines the following:

[a] Stannate precursor could not be synthesized.

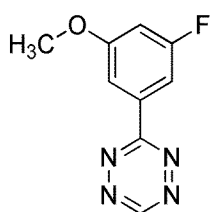
5 [b] No tetrazine formation detected.

[c] Decomposition during Cu-mediated ^{18}F -fluorination reaction.

[d] RCCs were determined by radio-HPLC ($n = 3$).

Example 59

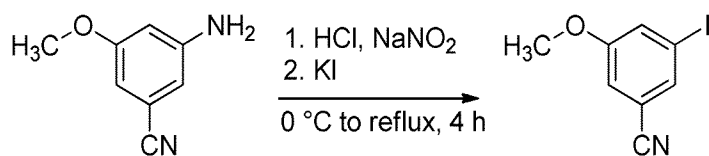
10



3-(3-fluoro-5-methoxyphenyl)-1,2,4,5-tetrazine (UB-048). The final compound was obtained from 3-fluoro-5-methoxybenzonitrile (0.60 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (85/15 n-Heptane/EtOAc) and recrystallized from n-Heptane to 0.21 g (26%) of UB-48 as a red solid. $R_f = 0.41$ (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.17 (s, 1H), 7.91 (d, $J = 2.0$ Hz, 1H), 7.87 (ddd, $J = 9.1$, 2.4, 1.4 Hz, 1H), 6.83 (dd, $J = 10.1$, 2.4 Hz, 1H), 3.86 (s, 3H); ^{13}C NMR (101 MHz, Chloroform- d) δ 165.63 (d, $J = 3.9$ Hz), 164.07 (d, $J = 246.5$ Hz), 161.75 (d, $J = 11.4$ Hz), 158.02, 133.96 (d, $J = 10.7$ Hz), 108.96 (d, $J = 2.8$ Hz), 107.73 (d, $J = 24.7$ Hz), 106.98 (d, $J = 24.9$ Hz), 55.96; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_9\text{H}_8\text{FN}_4\text{O}]^+$: 207.07; found 207.05

15

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Example 60

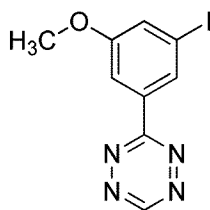
25

3-Iodo-5-methoxybenzonitrile (UB-224). Concentrated. HCl (3 mL) was added to a solution of aniline (1.00 g, 6.75 mmol) in water (3 mL) at 0 °C. To this was added a chilled solution of sodium nitrite (0.84 g, 12.15 mmol) in water (4 mL), dropwise, with vigorous mechanical stirring. Stirring was continued at 0 °C for 15 min. after the addition was complete, and then a solution of potassium iodide (2.24 g, 13.50 mmol) in water (4 mL) was added carefully. The

30

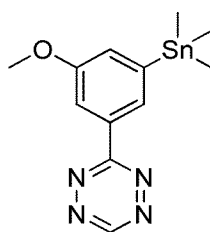
cooling bath was removed, and the reaction heated to reflux. When the production of purple vapor ceased, the mixture was cooled to rt and extracted with DCM (3×20 mL). The combined organic extracts were dried (MgSO₄), filtered, and concentrated under reduced pressure. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 0.60 g (34%) of UB-224 as a white solid. *R*_f = 0.34 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.48 (t, *J* = 1.4 Hz, 1H), 7.40 (dd, *J* = 2.4, 1.4 Hz, 1H), 7.04 (dd, *J* = 2.4, 1.4 Hz, 1H), 3.75 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 159.92, 132.68, 128.19, 116.85, 114.60, 94.14, 55.83.

Example 61



3-(3-iodo-5-methoxyphenyl)-1,2,4,5-tetrazine (UB-225). The final compound was obtained from 3-iodo-5-methoxybenzonitrile (0.52 g, 2.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (85/15 n-Heptane/EtOAc) and recrystallized from n-Heptane 0.19 g (30%) of UB-225 as a red solid. *R*_f = 0.25 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.17 (s, 1H), 8.49 (t, *J* = 1.4 Hz, 1H), 8.03 (dd, *J* = 2.4, 1.4 Hz, 1H), 7.44 (dd, *J* = 2.5, 1.5 Hz, 1H), 3.83 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 165.13, 160.72, 158.02, 134.10, 129.53, 128.40, 112.47, 94.96, 55.80.

Example 62



3-(3-methoxy-5-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (UB-229). The final compound was obtained from 55 mg (0.17 mmol) of the starting material, following the *General Procedure D.2*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.040 g (65%) of UB-229 as a purple solid. *R*_f = 0.41 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.21 (s, 1H), 8.35 (d, *J* = 1.5 Hz, 1H), 8.07 (dd, *J* = 2.7, 1.6 Hz, 1H), 7.32 (d, *J* = 2.7 Hz, 1H), 3.94 (s, 3H), 0.37 (s, 9H); ¹³C NMR (101 MHz,

Example 63 of UB-048:

The radiolabelling of UB-048 was provided as described in the Example 128, 129 and 130 and as shown in Table 2. In Table 2, the following letters in brackets defines the following:

[a] Stannate precursor could not be synthesized.

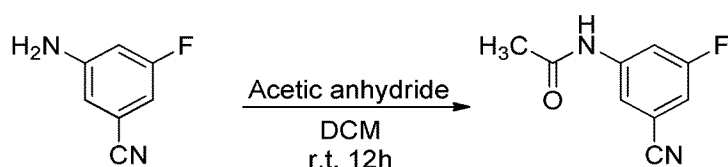
5 [b] No tetrazine formation detected.

[c] Decomposition during Cu-mediated ^{18}F -fluorination reaction.

[d] RCCs were determined by radio-HPLC (n = 3).

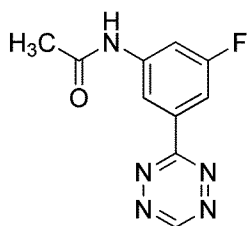
Example 64

10



N-(5-Cyano-3-fluorophenyl)acetamide (UB-149). To a solution of 3-amino-5-fluorobenzonitrile (0.82 g, 6.00 mmol) in DCM (30.0 mL) was added acetic anhydride (0.80 mL, 8.40 mmol). The mixture was stirred at room temperature for 12 h. The suspension was filtered, and the solvent removed under vacuum. Purification by flash chromatography (70/30 n-Heptane/EtOAc) afforded 0.92 g of N-(5-cyano-3-fluorophenyl)acetamide as a white solid. R_f = 0.31 (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 10.45 (s, 1H), 7.86 – 7.70 (m, 2H), 7.57 – 7.37 (m, 1H), 2.09 (s, 3H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 169.69, 162.24 (d, J = 244.3 Hz), 142.35 (d, J = 11.8 Hz), 118.65, 118.09 (d, J = 3.6 Hz), 113.70 (d, J = 25.5 Hz), 113.25 (d, J = 12.1 Hz), 110.95 (d, J = 26.2 Hz), 24.52.

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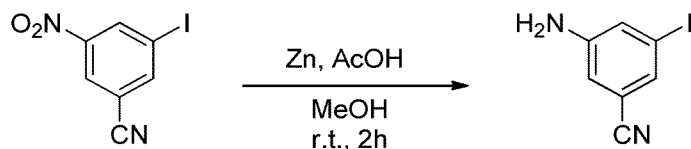
Example 65

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N-(3-Fluoro-5-(1,2,4,5-tetrazin-3-yl)phenyl)acetamide (UB-150). The final compound was obtained from N-(5-cyano-3-fluorophenyl)acetamide (0.58 g, 3.25 mmol) following *General Procedure C*. The crude was purified using flash chromatography (60/40 n-Heptane/EtOAc) to yield 0.19 g (25%) of a red solid. R_f = 0.25 (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 10.64 (s, 1H), 10.48 (s, 1H), 8.52 (t, J = 1.7 Hz, 1H), 7.98 – 7.81 (m, 2H), 2.12 (s, 3H);

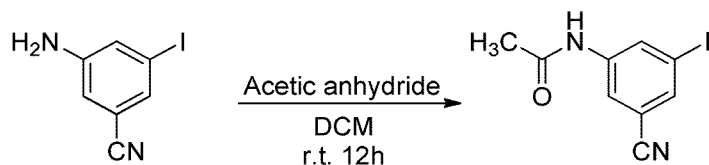
^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 169.54, 165.03 (d, $J = 3.8$ Hz), 163.08 (d, $J = 242.2$ Hz), 158.83, 142.45 (d, $J = 11.5$ Hz), 134.61 (d, $J = 10.1$ Hz), 114.40 (d, $J = 2.6$ Hz), 109.87 (d, $J = 26.6$ Hz), 108.74 (d, $J = 24.4$ Hz), 24.58; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{10}\text{H}_9\text{FN}_5\text{O}]^+$: 234.08; found 234.10.

5

Example 66

3-Amino-5-iodobenzonitrile (UB-210). To a solution of 3-iodo-5-nitrobenzonitrile (0.500 g, 1.82 mmol) and Zn (0.58 g, 8.87 mmol) in MeOH (20 mL) was added dropwise 1 mL of acetic acid. The reaction was stirred at room temperature for 2 h and then concentrated under reduced pressure. Purification by flash chromatography afforded 0.250 g (56%) of UB-210 as a white solid. $R_f = 0.22$ (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 7.24 (d, $J = 1.4$ Hz, 1H), 7.15 (t, $J = 1.9$ Hz, 1H), 6.81 – 6.73 (m, 1H), 3.81 (s, 2H); ^{13}C NMR (101 MHz, Chloroform- d) δ 147.74, 129.88, 127.63, 117.39, 116.80, 114.45, 94.45.

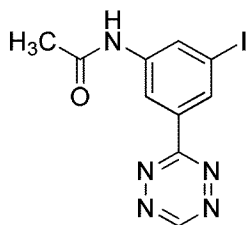
15

Example 67

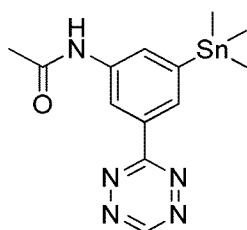
N-(5-Cyano-3-iodophenyl)acetamide (UB-149). To a solution of 3-amino-5-iodobenzonitrile (0.20 g, 0.81 mmol) in DCM (10.0 mL) was added acetic anhydride (0.1 mL, 1.15 mmol). The mixture was stirred at room temperature for 12 h. The suspension was filtered, and the solvent removed under vacuum. Purification by flash chromatography (70/30 n-Heptane/EtOAc) afforded 0.21 (90%) of UB-210 as a white solid. $R_f = 0.29$ (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, Methanol- d_4) δ 8.13 (t, $J = 1.8$ Hz, 1H), 7.87 (t, $J = 1.7$ Hz, 1H), 7.66 (t, $J = 1.5$ Hz, 1H), 2.04 (s, 3H); ^{13}C NMR (101 MHz, Methanol- d_4) δ 170.51, 140.51, 134.84, 132.16, 121.42, 116.54, 113.80, 93.18, 22.51.

25

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Example 68

N-(3-iodo-5-(1,2,4,5-tetrazin-3-yl)phenyl)acetamide (UB-216). The final compound was obtained from N-(5-cyano-3-iodophenyl)acetamide (0.18 g, 0.63 mmol) following *General Procedure C*. The crude was purified using flash chromatography (60/40 n-Heptane/EtOAc) to yield 0.055 g (26%) of UB-216 as a red solid. *R_f* = 0.21 (n-Heptane:40%EtOAc); ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.63 (s, 1H), 10.35 (s, 1H), 8.73 (t, *J* = 1.7 Hz, 1H), 8.44 (t, *J* = 1.5 Hz, 1H), 8.38 (t, *J* = 1.8 Hz, 1H), 2.11 (s, 3H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 169.44, 164.69, 158.81, 141.92, 134.71, 131.04, 130.64, 117.61, 95.93, 24.59.

Example 69

N-(3-(1,2,4,5-tetrazin-3-yl)-5-(trimethylstannyl)phenyl)acetamide (UB-220). The final compound was obtained from 55 mg (0.17 mmol) of the starting material, following the *General Procedure D.2*. The crude was purified using flash chromatography (70/30 n-Heptane/EtOAc) to yield 0.025 g (41%) of UB-220 as a purple oil. *R_f* = 0.35 (n-Heptane:50%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.20 (s, 1H), 8.56 (t, *J* = 2.1 Hz, 1H), 8.44 (s, 1H), 8.03 (d, *J* = 2.2 Hz, 1H), 7.63 (s, 1H), 2.24 (s, 3H), 0.36 (s, 9H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 168.68, 166.46, 157.80, 145.49, 138.40, 131.68, 131.52, 131.18, 119.43, 24.60, -9.30; HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₃H₁₈N₅SnO]⁺: 380.05; Found: 380.09.

Example 70 of UB-150:

The radiolabelling of UB150 was provided as described in the Example 128, 129 and 130 and as shown in Table 2. In Table 2, the following letters in brackets defines the following:

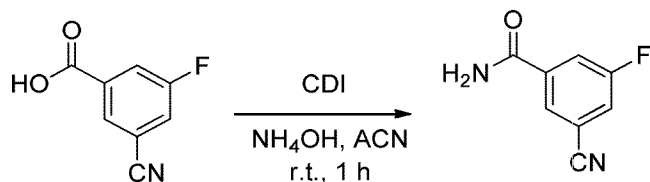
[a] Stannate precursor could not be synthesized.

[b] No tetrazine formation detected.

[c] Decomposition during Cu-mediated ¹⁸F-fluorination reaction.

[d] RCCs were determined by radio-HPLC (n = 3).

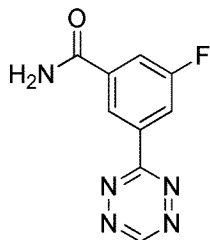
Example 71



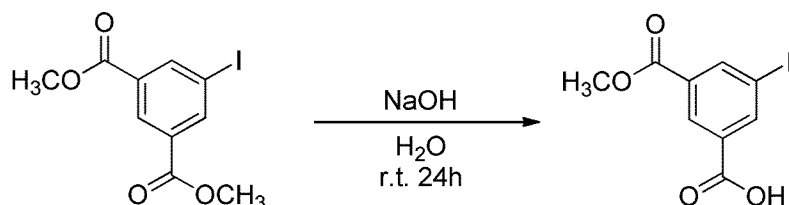
5 **3-Cyano-5-fluorobenzamide (UB-69).** To a solution of 5-cyano-3-fluorobenzoic acid (0.99 g, 6.0 mmol) in acetonitrile (20 ml) was added 1,1'-carbonyldiimidazole (1.46 g, 9.0 mmol). The mixture was stirred at room temperature for 45 min, before addition of aqueous ammonium hydroxide solution (35%, 20 ml). The reaction mixture was stirred for 45 min and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the title
10 compound 0.77 g (78%) of UB-69 as a white solid. *R_f* = 0.41 (n-Heptane:40%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.21 (s, 1H), 8.16 (d, *J* = 1.5 Hz, 1H), 8.05 (ddd, *J* = 8.4, 2.6, 1.3 Hz, 1H), 8.01 (ddd, *J* = 9.6, 2.5, 1.4 Hz, 1H), 7.78 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.12 (d, *J* = 2.4 Hz), 162.04 (d, *J* = 247.5 Hz), 138.39 (d, *J* = 7.3 Hz), 128.11 (d, *J* = 3.1 Hz), 122.37 (d, *J* = 25.7 Hz), 120.16 (d, *J* = 22.9 Hz), 117.69 (d, *J* = 3.1 Hz), 113.52 (d, *J* = 9.9 Hz).

15

Example 72

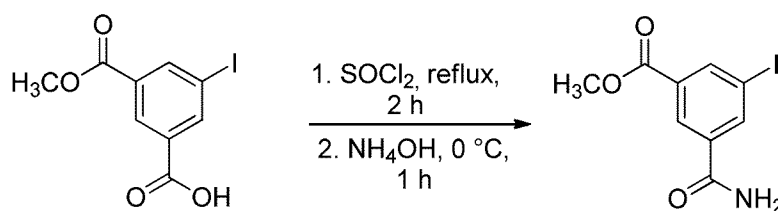


20 **3-Fluoro-5-(1,2,4,5-tetrazin-3-yl)benzamide (UB-70).** The final compound was obtained from 3-cyano-5-fluorobenzamide (0.75 g, 4.57 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 CH₂Cl₂/MeOH) to yield a yield 0.36 g (36%) of UB-70 as a pink solid. *R_f* = 0.31 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.69 (s, 1H), 8.88 (s, 1H), 8.48 – 8.20 (m, 2H), 8.16 – 7.92 (m, 1H), 7.71 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 166.08 (d, *J* = 2.3 Hz), 164.94 (d, *J* = 3.3 Hz), 162.85 (d, *J* = 245.6
25 Hz), 158.89, 138.32 (d, *J* = 6.9 Hz), 134.93 (d, *J* = 8.2 Hz), 123.56 (d, *J* = 2.9 Hz), 118.85 (d, *J* = 23.0 Hz), 117.31 (d, *J* = 24.1 Hz); HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₉H₇FN₅O]⁺: 220.06; found 220.09.

Example 73

3-Iodo-5-(methoxycarbonyl)benzoic acid (UB-277). The preparation of this intermediate,

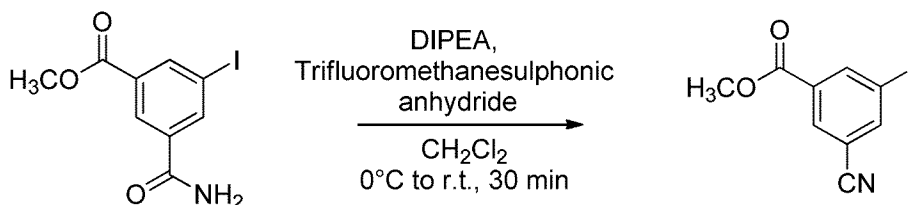
was performed using a method described previously.^[19] To a solution of dimethyl 5-iodoisophthalate (12.8 g, 40 mmol), methanol (80 mL), and CH₂Cl₂ (40 mL) was added NaOH (1.68 g, 42 mmol). The mixture was allowed to stir at room temperature for 24 hours. The solvents were removed under reduced pressure. Lots of white precipitate formed when water (9 mL), dichloromethane (10 mL), and ethyl acetate (10 mL) were added while stirring, which was collected by filtration, well washed with a mixture of dichloromethane (10 mL) and ethyl acetate (10 mL), and then with water (10 mL). After transferring the solid (mono sodium salt) to a separatory funnel, ethyl acetate (80 mL) and conc. HCl (3 mL) diluted with water (20 mL) were successively added. The mixture was vigorously shaken until the solid was disappeared. Then the organic layer was separated, and the aqueous layer was extracted by ethyl acetate (25 mL). The organic layers were combined and washed by brine (20 mL), dried over MgSO₄, filtered, and concentrated. The solid obtained was washed recrystallized from MeOH to give 10.3 g (84%) of UB-277 as a white solid. *R_f* = 0.33 (CH₂Cl₂:5%MeOH:0.1%AcOH); ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.57 (s, 1H), 8.49 – 8.27 (m, 3H), 3.89 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.55, 164.68, 142.24, 141.62, 133.67, 132.31, 129.36, 95.42, 53.15.

Example 74

Methyl 3-carbamoyl-5-iodobenzoate (UB-280). A solution of 3-methoxycarbonyl-5-iodobenzoic acid (10.3 g, 33.65 mmol) in thionyl chloride (30.0 mL) was heated for 2 hours at 60 °C. The reaction mixture was cooled and concentrated under reduced pressure. The intermediate acid chloride was then diluted with tetrahydrofuran (40 mL) and cooled to 0 °C. The mixture was then treated with a solution of 2M ammonia (60 mL, 120 mmol, methanol) and the reaction stirred for 1 hour at 0 °C. The mixture was then filtered, and the solvent

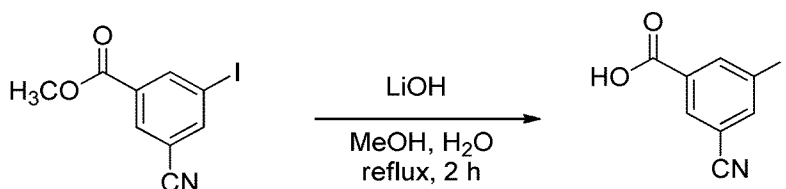
removed under reduced pressure. Recrystallization from methanol afforded 7.70 g (75%) of UB-280 as a white solid. R_f = 0.25 (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.47 (t, J = 1.6 Hz, 1H), 8.44 (d, J = 1.6 Hz, 1H), 8.35 (t, J = 1.6 Hz, 1H), 8.24 (s, 1H), 7.61 (s, 1H), 3.89 (s, 3H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 165.86, 164.96, 140.72, 140.21, 136.99, 132.10, 128.09, 95.18, 53.06.

Example 75



Methyl 3-cyano-5-iodobenzoate (UB-284). At a temperature of about 0°C, a solution of 2.8 ml (16.5 mmol) of trifluoromethanesulphonic anhydride in 50 ml of dichloromethane was added dropwise to a solution of 2.80 g (9.18 mmol) of methyl 3-carbamoyl-5-iodobenzoate and 8 ml (45.9 mmol) of N,N-diisopropylethylamine in 150 ml of dichloromethane. After a reaction time of 30 min at 0°C, 50 ml of saturated aqueous sodium bicarbonate solution were added, and the mixture was stirred vigorously at room temperature for 10 minutes. The organic phase was separated off, dried over anhydrous MgSO_4 , filtered and freed from the solvent on a rotary evaporator. Purification by flash chromatography (80/20 Heptane/EtOAc) afforded 2.4 g (91%) of UB-284 as a white solid. R_f = 0.5 (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 8.58 (s, 1H), 8.27 (s, 1H), 8.14 (s, 1H), 3.96 (s, 3H); ^{13}C NMR (101 MHz, Chloroform- d) δ 163.72, 144.07, 142.59, 132.70, 132.25, 116.25, 114.55, 93.68, 52.99.

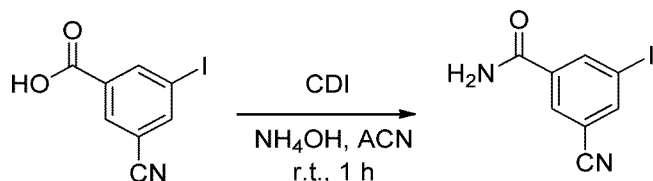
Example 76



3-Cyano-5-iodobenzoic acid (UB-285). A solution of methyl 3-cyano-5-iodobenzoate (2.36 g, 8.22 mmol) in THF (25 mL) was treated with 0.5M LiOH (20 mL, 9.86 mmol) and methanol. The reaction mixture was heated at reflux for 1 hour. The solvent was concentrated in vacuo and the mixture treated with 1N HCl. The resulting white precipitate was filtered, and the filtrate was extracted with dichloromethane. The residue and the extracted filtrate were combined and concentrated in vacuo to afford 2.1 g (94%) UB-285 as a white solid. R_f = 0.33 (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.73 (s, 1H), 8.65 (s, 1H), 8.35 (s,

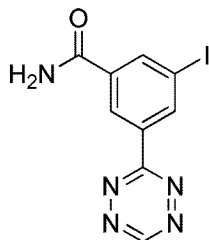
1H), 8.22 (s, 1H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 168.43, 145.01, 143.10, 132.80, 131.74, 116.04, 114.77, 93.84.

Example 77

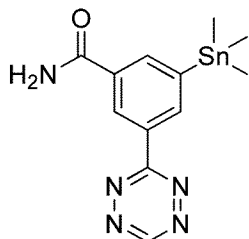


3-Cyano-5-iodobenzamide (UB-263). To a solution of 5-iodo-3-cyanobenzoic acid (0.40 g, 1.46 mmol) in acetonitrile (10 ml) was added 1,1'-carbonyldiimidazole (0.36 g, 2.20 mmol). The mixture was stirred at room temperature for 45 min, before addition of ammonium hydroxide solution (80%, 5 ml). The reaction mixture was stirred for 45 min and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the title compound 0.35 g (88%) of UB-263 as a white solid. *R*_f = 0.44 (n-Heptane:40%EtOAc); ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.41 (s, 1H), 8.18 (s, 1H), 8.11 (s, 1H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 166.85, 142.88, 140.78, 136.39, 130.23, 116.17, 113.99, 93.36.

Example 78



3-Iodo-5-(1,2,4,5-tetrazin-3-yl)benzamide (UB-265). The final compound was obtained from 3-cyano-5-iodobenzamide (0.21 g, 0.77 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 CH₂Cl₂/MeOH) to yield 0.07 g (28%) of UB-265 as a pink solid. *R*_f = 0.41 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.68 (s, 1H), 8.99 (d, *J* = 1.7 Hz, 1H), 8.89 (d, *J* = 1.7 Hz, 1H), 8.55 (d, *J* = 1.7 Hz, 1H), 8.34 (s, 1H), 7.66 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.96, 164.64, 158.84, 140.13, 138.75, 137.55, 134.60, 126.77, 95.96.

Example 79

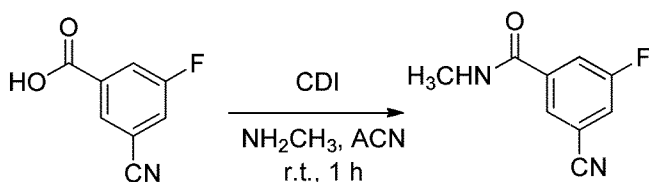
3-(1,2,4,5-tetrazin-3-yl)-5-(trimethylstannyl)benzamide (UB-272). The final compound was obtained from 55 mg (0.17 mmol) of the starting material, following the *General Procedure D.2*.

- 5 The crude was purified using flash chromatography (40/60 n-Heptane/EtOAc) to yield 0.025 g (41%) of UB-220 as a purple oil. *R_f* = 0.42 (n-Heptane:60%EtOAc); ¹H NMR (600 MHz, Methanol-*d*₄) δ 10.28 (s, 1H), 8.76 (dd, *J* = 1.8, 0.9 Hz, 1H), 8.20 (dd, *J* = 1.9, 0.8 Hz, 1H), 0.31 (s, 9H); ¹³C NMR (151 MHz, Methanol-*d*₄) δ 170.47, 166.26, 158.03, 144.81, 138.52, 137.69, 134.02, 131.68, 126.83, -11.10; HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₁₂H₁₆N₅SnO]⁺: 366.04; Found: 366.08.

Example 80 of UB-70:

The radiolabelling of UB-70 was provided as described in Example 128, 129 and 130 and as shown in Table 2. In Table 2, the following letters in brackets defines the following:

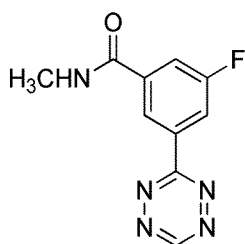
- 15 [a] Stannate precursor could not be synthesized.
[b] No tetrazine formation detected.
[c] Decomposition during Cu-mediated ¹⁸F-fluorination reaction.
[d] RCCs were determined by radio-HPLC (*n* = 3).

Example 81

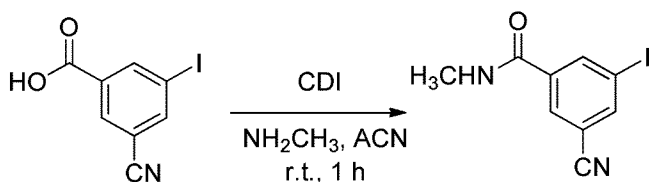
- 5-Cyano-3-fluoro-N-methylbenzamide (UB-199).** To a solution of 5-cyano-3-fluorobenzoic acid (0.99 g, 6.0 mmol) in acetonitrile (20 ml) was added 1,1'-carbonyldiimidazole (1.46 g, 9.0 mmol). The mixture was stirred at room temperature for 45 min, before addition of aqueous methylamine solution (40%, 20 ml). The reaction mixture was stirred for 45 min and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the title compound 0.81 g (76%) of UB199 as a white solid. *R_f* = 0.32 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.72 (d, *J* = 5.8 Hz, 1H), 8.11 (t, *J* = 1.5 Hz, 1H), 8.05 (ddd, *J* = 8.1,

2.7, 1.4 Hz, 1H), 7.97 (ddd, $J = 9.5, 2.7, 1.5$ Hz, 1H), 2.81 (d, $J = 4.6$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 163.96 (d, $J = 2.5$ Hz), 162.02 (d, $J = 247.5$ Hz), 138.49 (d, $J = 7.4$ Hz), 127.76 (d, $J = 3.3$ Hz), 122.17 (d, $J = 25.6$ Hz), 119.85 (d, $J = 23.1$ Hz), 117.66 (d, $J = 3.4$ Hz), 113.55 (d, $J = 10.0$ Hz), 26.83.

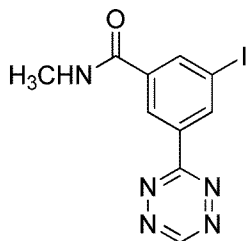
5

Example 82

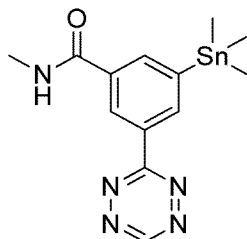
3-Fluoro-N-methyl-5-(1,2,4,5-tetrazin-3-yl)benzamide (UB-200). The final compound was obtained from 5-cyano-3-fluoro-N-methylbenzamide (0.62 g, 3.48 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) to afford 0.28 g (34%) of UB-200 as a pink solid. $R_f = 0.31$ (n-Heptane:60%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 10.70 (s, 1H), 8.87 (s, 2H), 8.39 (dd, $J = 9.3, 1.9$ Hz, 1H), 7.99 (dt, $J = 9.5, 2.0$ Hz, 1H), 2.85 (d, $J = 4.5$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 164.88 (d, $J = 11.1$ Hz), 162.89 (d, $J = 245.8$ Hz), 158.92, 138.41 (d, $J = 7.5$ Hz), 135.00 (d, $J = 8.2$ Hz), 133.13, 123.08, 118.56 (d, $J = 22.8$ Hz), 117.16 (d, $J = 24.0$ Hz), 26.89; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{10}\text{H}_9\text{FN}_5\text{O}]^+$: 234.08; found 234.06.

Example 83

5-Cyano-3-iodo-N-methylbenzamide (UB-262). To a solution of 5-cyano-3-iodobenzoic acid (0.40 g, 1.46 mmol) in acetonitrile (10 ml) was added 1,1'-carbonyldiimidazole (0.36 g, 2.20 mmol). The mixture was stirred at room temperature for 45 min, before addition of aqueous Methylamine solution (80%, 5 ml). The reaction mixture was stirred for 45 min and ice-cold water (15 ml) was added. The precipitate was collected by filtration and dried to give the title compound 0.41 g (98%) of UB-262 as a white solid. $R_f = 0.55$ (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, Methanol- d_4) δ 8.45 (d, $J = 1.7$ Hz, 1H), 8.27 (d, $J = 1.9$ Hz, 1H), 8.15 (s, 1H), 2.93 (s, 3H); ^{13}C NMR (101 MHz, Methanol- d_4) δ 165.38, 142.63, 140.38, 136.78, 129.83, 116.20, 113.97, 93.44, 25.67.

Example 84

3-iodo-N-methyl-5-(1,2,4,5-tetrazin-3-yl)benzamide (UB-264). The final compound was
 5 obtained from 5-cyano-3-iodo-N-methylbenzamide (0.38 g, 1.32 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 CH₂Cl₂/MeOH) to yield 0.11 g (24%) of UB254 as a pink solid. *R_f* = 0.45 (n-Heptane:60%EtOAc); ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.68 (s, 1H), 8.97 (s, 1H), 8.89 (s, 1H), 8.84 (q, *J* = 4.5 Hz, 1H), 8.51 (s, 1H), 2.83 (d, *J* = 4.5 Hz, 3H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 164.67, 164.62, 158.89, 139.78,
 10 138.57, 137.63, 134.64, 126.31, 96.04, 26.89.

Example 85

N-methyl-3-(1,2,4,5-tetrazin-3-yl)-5-(trimethylstannyl)benzamide (UB-269). The final
 15 compound was obtained from 50 mg (0.14 mmol) of the starting material, following the *General Procedure D.2*. The crude was purified using flash chromatography (60/40 n-Heptane/EtOAc) to yield 0.035 g (63%) of UB269 as a purple oil. *R_f* = 0.46 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.25 (s, 1H), 8.89 – 8.80 (m, 2H), 8.26 (dd, *J* = 1.9, 0.9 Hz, 1H), 6.37 (s, 1H), 3.08 (d, *J* = 4.8 Hz, 3H), 0.39 (s, 9H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 167.68,
 20 166.22, 157.96, 145.55, 139.44, 138.02, 134.89, 130.88, 125.60, 26.97, -9.23; HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₃H₁₈N₅SnO]⁺: 380.05; Found: 380.02.

Example 86 of UB-200:

The radiolabelling of UB-007 was provided as described in the Example 128, 129 and 130 and
 25 as shown in Table 2. In Table 2, the following letters in brackets defines the following:

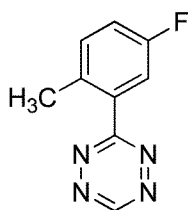
[a] Stannate precursor could not be synthesized.

[b] No tetrazine formation detected.

[c] Decomposition during Cu-mediated ^{18}F -fluorination reaction.

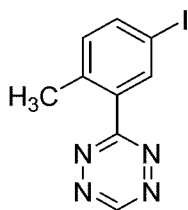
[d] RCCs were determined by radio-HPLC ($n = 3$).

Example 87

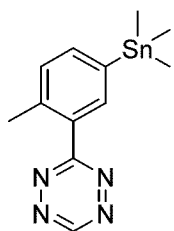


3-(3-fluoro-6-methylphenyl)-1,2,4,5-tetrazine (RGV-117). The final compound was obtained from 5-fluoro-2-methylbenzonitrile (0.54 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield after crystallization with n-Heptane 0.12 g (16%) of a red solid. $R_f = 0.37$ (n-Heptane:10%EtOAc); ^1H NMR (600 MHz, Chloroform- d) δ 10.23 (s, 1H), 7.72 (dd, $J = 8.5, 3.2$ Hz, 1H), 7.32 – 7.27 (m, 1H), 7.08 (dd, $J = 9.1, 4.2$ Hz, 1H), 3.90 (s, 3H); ^{13}C NMR (151 MHz, Chloroform- d) δ 167.77 (d, $J = 2.2$ Hz), 157.08 (d, $J = 240.5$ Hz), 156.99, 154.97 (d, $J = 2.1$ Hz), 122.91 (d, $J = 7.8$ Hz), 120.08 (d, $J = 22.9$ Hz), 118.48 (d, $J = 25.3$ Hz), 113.94 (d, $J = 7.9$ Hz), 56.96.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_9\text{H}_7\text{FN}_4]^+$: 191.07; Found 191.09.

Example 88



3-(5-iodo-2-methylphenyl)-1,2,4,5-tetrazine (RGV_125). The final compound was obtained from 3-iodo-5-methylbenzonitrile (0.97 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield after crystallization with n-Heptane 0.21 g (17%) of a red solid. $R_f = 0.42$ (n n-Heptane:10%EtOAc).; ^1H NMR (600 MHz, Chloroform- d) δ 10.26 (s, 1H), 8.43 (s, 1H), 7.81 (d, $J = 8.1$ Hz, 1H), 7.15 (d, $J = 8.1$ Hz, 1H), 2.59 (s, 3H); ^{13}C NMR (151 MHz, Chloroform- d) δ 168.60, 157.06, 140.71, 139.61, 138.56, 133.95, 133.52, 90.88, 21.28.

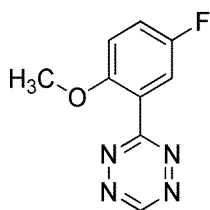
Example 89

3-(2-methyl-5-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_139). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General Procedure*

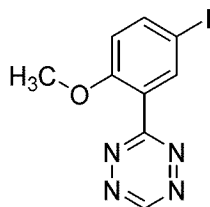
D.2. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.10 g (18%) of a pink solid. *R_f* = 0.32 (n-Heptane:20%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.23 (s, 1H), 8.15 (s, 1H), 7.63 (d, *J* = 7.4 Hz, 1H), 7.38 (d, *J* = 7.4 Hz, 1H), 2.62 (s, 3H), 0.33 (s, 9H); ¹³C NMR (151 MHz, Chloroform-*d*) δ 170.16, 156.91, 140.46, 139.45, 138.73, 138.49, 131.61, 131.48, 21.39, -9.27.; HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₂H₁₆N₄Sn]⁺: 337.05;

Found: 337.1.

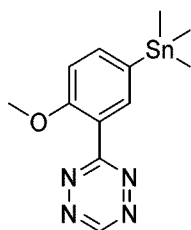
Chloroform-*d*) δ 166.64, 157.77, 145.64, 132.10, 127.94, 127.56, 111.71, 55.45, -9.36; HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₂H₁₇N₄SnO]⁺: 353.04; Found: 353.07.

Example 90

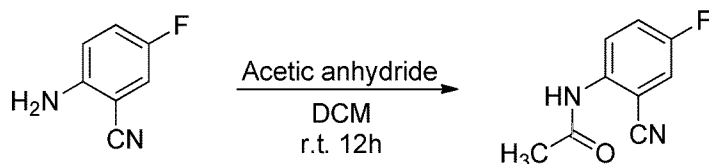
3-(3-fluoro-6-methoxyphenyl)-1,2,4,5-tetrazine (RGV-116). The final compound was obtained from 5-fluoro-2-methoxybenzonitrile (0.60 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield after crystallization with n-Heptane 0.13 g (16%) as a red solid. *R_f* = 0.18 (n-Heptane:10%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.23 (s, 1H), 8.26 (s, 1H), 8.12 (d, *J* = 9.4 Hz, 1H), 7.17 (d, *J* = 9.1 Hz, 1H), 2.50 (s, 3H); ¹³C NMR (151 MHz, Chloroform-*d*) δ 165.99 (d, *J* = 3.4 Hz), 163.45 (d, *J* = 246.8 Hz), 158.11, 141.97 (d, *J* = 7.8 Hz), 133.45 (d, *J* = 8.9 Hz), 124.81, 120.95 (d, *J* = 21.2 Hz), 112.44 (d, *J* = 24.2 Hz), 21.60; HPLC-MS [M+H]⁺ *m/z* calc. for [C₉H₇FN₄O]⁺: 207.07; Found: 207.9.

Example 91

3-(5-iodo-2-methoxyphenyl)-1,2,4,5-tetrazine (RGV_105). The final compound was obtained from 3-iodo-5-methoxybenzonitrile (1.03 g, 4.00 mmol) following *General Procedure*
 5 C. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield after crystallization with n-Heptane 0.19 g (15%) as a red solid. *Rf* = 0.21 (n-Heptane:20%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.21 (s, 1H), 8.21 (s, 1H), 7.82 (dd, *J* = 8.8, 2.3 Hz, 1H), 6.88 (d, *J* = 8.8 Hz, 1H), 3.87 (s, 3H); ¹³C NMR (151 MHz, CDCl₃) δ 167.31, 158.39, 156.94, 142.10, 140.14, 124.08, 114.62, 82.71, 56.35.

Example 92

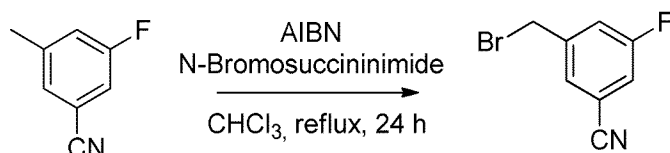
3-(2-methoxy-5-(trimethylstannyl)phenyl)-1,2,4,5-tetrazine (RGV_110). The final compound was obtained from 50 mg (0.17 mmol) of the starting material, following the *General*
 15 *Procedure D.1*. The crude was purified using flash chromatography (90/10 n-Heptane/EtOAc) to yield 0.23 g (40%) of a pink solid. *Rf* = 0.16 (n-Heptane:10%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.21 (s, 1H), 8.11 – 7.92 (m, 1H), 7.75 – 7.58 (m, 1H), 7.19 – 7.05 (m, 1H), 3.90 (s, 3H), 0.32 (s, 9H); ¹³C NMR (151 MHz, CDCl₃) δ 168.91, 158.89, 156.86, 141.13, 139.19, 133.70, 122.09, 112.20, 56.11, -9.19; HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₁₂H₁₆N₄OSn]⁺: 353.04; Found: 353.1.

Example 93

N-(2-cyano-4-fluorophenyl)acetamide (UB-154). To a solution of the 2-amino-5-fluorobenzonitrile (0.82 g, 6.00 mmol) in DCM (30.0 mL) was added acetic anhydride (0.80 mL,
 25

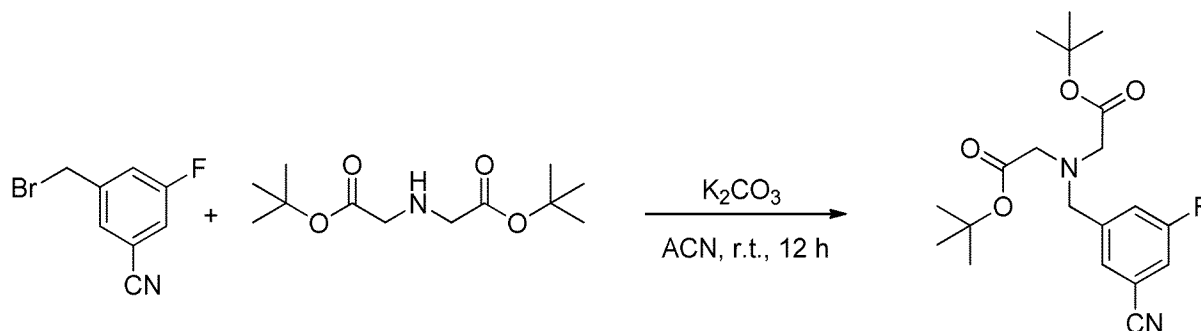
8.40 mmol). The mixture was stirred at room temperature for 12 hours. The suspension was filtered, and the solvent removed under vacuum. Purification by flash chromatography (70/30 Heptane/EtOAc) afforded 0.81 g (76%) of UB-154 as a white solid. R_f = 0.41 (n-Heptane:40%EtOAc); ^1H NMR (400 MHz, DMSO- d_6) δ 10.16 (s, 1H), 7.81 (dd, J = 8.4, 1.8 Hz, 1H), 7.59 (d, J = 1.7 Hz, 1H), 7.57 (d, J = 1.7 Hz, 1H), 2.09 (s, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 169.22, 158.86 (d, J = 244.3 Hz), 137.55 (d, J = 3.0 Hz), 128.41 (d, J = 8.7 Hz), 121.74 (d, J = 22.4 Hz), 120.01 (d, J = 26.0 Hz), 116.21 (d, J = 2.7 Hz), 109.34, 23.49, 164.47, 164.13, 149.66, 136.88, 132.80, 132.00, 131.62, 129.46, 128.11, 127.08, -9.32.; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{12}\text{H}_{16}\text{N}_4\text{OSn}]^+$: 353.04; Found: 353.1.

Example 94



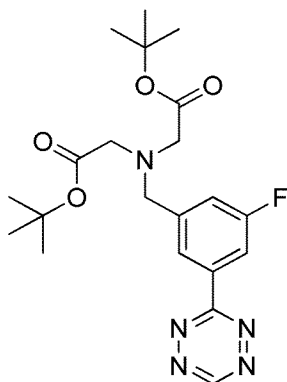
3-(Bromomethyl)-5-fluorobenzonitrile (UB-98). To a solution of 3-fluoro-5-methylbenzonitrile (2.61 g, 19.24 mmol) and N-bromosuccinimide (5.13 g, 28.86 mmol) in CHCl_3 was added AIBN (1.26 g, 7.69 mmol). The reaction was refluxed for 24 h. The solvent was removed under vacuum and the crude purified by flash chromatography (heptane/EtOAc 95/5) to give 2.10 g (51%) of UMB-98 as a colorless oil. R_f = 0.32 (n-Heptane:5%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 7.50 (t, J = 1.5 Hz, 1H), 7.38 (dt, J = 8.9, 2.0 Hz, 1H), 7.30 (ddd, J = 7.9, 2.5, 1.3 Hz, 1H), 4.46 (s, 2H); ^{13}C NMR (101 MHz, Chloroform- d) δ 162.12 (d, J = 251.3 Hz), 141.93 (d, J = 8.0 Hz), 128.52 (d, J = 3.4 Hz), 121.06 (d, J = 22.0 Hz), 118.97 (d, J = 24.7 Hz), 117.07 (d, J = 3.3 Hz), 114.26 (d, J = 9.9 Hz), 30.32 (d, J = 1.9 Hz).

Example 95



Di-tert-butyl 2,2'-((3-cyano-5-fluorobenzyl)azanediyl)diacetate (UB-100). To a solution of 3-fluoro-5-bromomethylbenzonitrile (1.09 g, 5.10 mmol) in CH_3CN (30 mL) was added K_2CO_3 (1.06 g, 7.65 mmol) and di-tert-butyl iminodiacetate (1.50 g, 6.12 mmol). The reaction

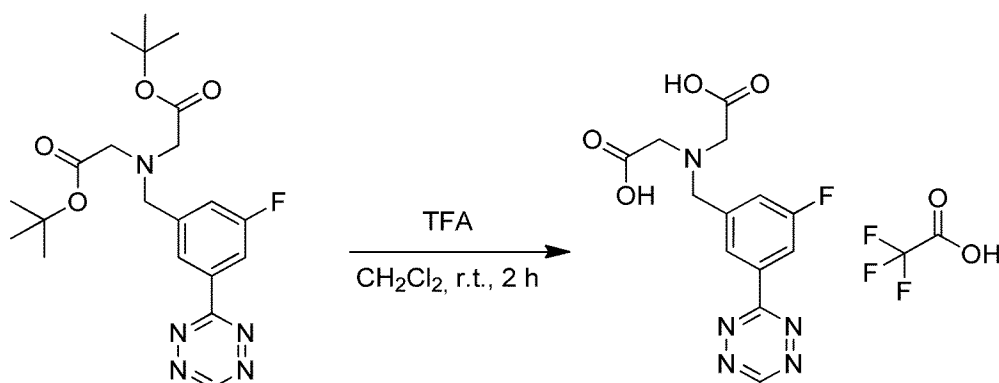
mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 1.72 g (89%) of UB-100 as a white solid. *R_f* = 0.24 (n-Heptane:10%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.53 (s, 1H), 7.51 – 7.42 (m, 1H), 7.23 (ddd, *J* = 7.8, 2.5, 1.4 Hz, 1H), 3.93 (s, 2H), 3.39 (s, 4H), 1.46 (s, 18H);



10 Example 96

Di-tert-butyl 2,2'-((3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-104).

The compound was obtained from di-tert-butyl 2,2'-((3-cyano-5-fluorobenzyl)azanediyl)diacetate (1.70 g, 4.49 mmol) following *General Procedure C*. The crude was purified using flash chromatography (heptane/EtOAc 95/5) to yield 0.11 g (24%) of UB-104 as a red solid. *R_f* = 0.39 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.17 (s, 1H), 8.34 (d, *J* = 1.4 Hz, 1H), 8.14 (ddd, *J* = 9.2, 2.5, 1.5 Hz, 1H), 7.55 – 7.45 (m, 1H), 3.98 (s, 2H), 3.40 (s, 4H), 1.41 (s, 18H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 170.25, 165.78 (d, *J* = 3.2 Hz), 163.58 (d, *J* = 247.5 Hz), 157.97, 143.30 (d, *J* = 7.1 Hz), 133.46 (d, *J* = 8.7 Hz), 124.18 (d, *J* = 2.7 Hz), 120.64 (d, *J* = 21.8 Hz), 114.15 (d, *J* = 24.5 Hz), 81.34, 56.96 (d, *J* = 1.8 Hz), 55.35, 28.17.

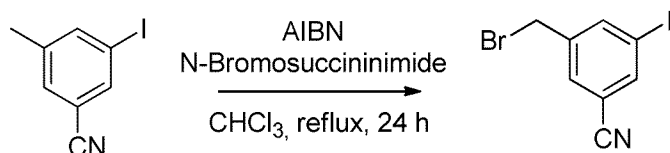
Example 97

1-Carboxy-N-(carboxymethyl)-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (UB-108).

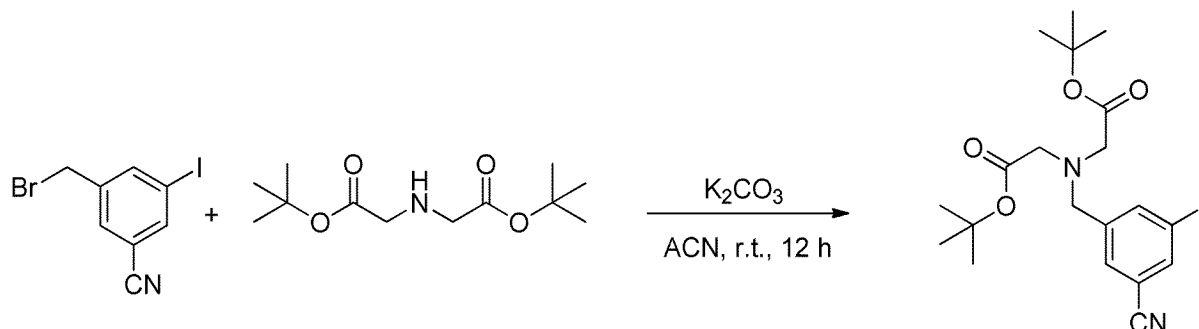
To a solution of di-tert-butyl 2,2'-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.15 g, 0.36 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 hours. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.08 g (51%) of UMB-108 as a pink solid. ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.42 (s, 1H), 8.60 (d, *J* = 1.4 Hz, 1H), 8.41 – 8.32 (m, 1H), 7.73 – 7.64 (m, 1H), 5.11 (s, 7H), 4.59 (s, 2H), 4.11 (s, 4H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 168.68, 165.12 (d, *J* = 3.2 Hz), 163.27 (d, *J* = 247.6 Hz), 158.24, 135.40 (d, *J* = 7.7 Hz), 135.10 (d, *J* = 8.6 Hz), 126.05 (d, *J* = 3.0 Hz), 121.71 (d, *J* = 22.7 Hz), 115.46 (d, *J* = 24.4 Hz), 57.85, 53.62; HPLC-MS [M+H]⁺ *m/z* calc.

for [C₁₃H₁₃FN₅O₄]⁺: 322.09; Found: 323.11.

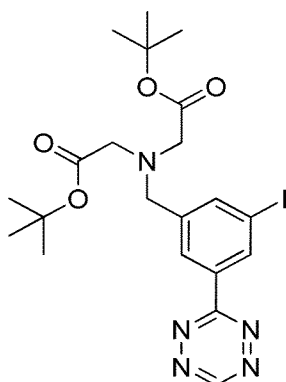
Figure 5 displays the whole synthesis scheme. Figure 7 displays the NMR chromatogram.

Example 98

3-(Bromomethyl)-5-iodobenzonitrile (UB-142). To a solution of 3-iodo-5-methylbenzonitrile (2.50 g, 10.28 mmol) and N-bromosuccinimide (2.28 g, 12.86 mmol) in CHCl₃ (40 mL) was added AIBN (0.67 g, 4.11 mmol). The reaction was refluxed for 24 h. The solvent was removed under vacuum and the crude purified by flash chromatography (heptane/EtOAc 95/5) to give 1.61 g (49%) of UMB-142 as a white solid. *R_f* = 0.28 (n-Heptane:5%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.96 (d, *J* = 1.6 Hz, 1H), 7.89 (d, *J* = 1.6 Hz, 1H), 7.64 (t, *J* = 1.6 Hz, 1H), 4.38 (s, 2H) ¹³C NMR (101 MHz, Chloroform-*d*) δ 142.20, 140.90, 140.12, 131.67, 116.55, 114.56, 94.05, 29.90.

Example 99

Di-tert-butyl 2,2'-((3-cyano-5-iodobenzyl)azanediyl)diacetate (UB-152). To a solution of 3-(bromomethyl)-5-iodobenzonitrile (1.00 g, 3.10 mmol) in CH₃CN (30 mL) was added K₂CO₃ (0.64 g, 7.65 mmol) and the corresponding amine (0.91 g, 3.72 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 1.35 g (89%) of UB-152 as a colorless oil. *R_f* = 0.31 (n-Heptane:5%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.96 (s, 1H), 7.80 (s, 1H), 7.65 (s, 1H), 3.83 (s, 2H), 3.33 (s, 4H), 1.40 (s, 18H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 169.95, 142.47, 142.14, 139.29, 131.57, 117.13, 114.11, 93.93, 81.52, 56.16, 55.23, 28.17.

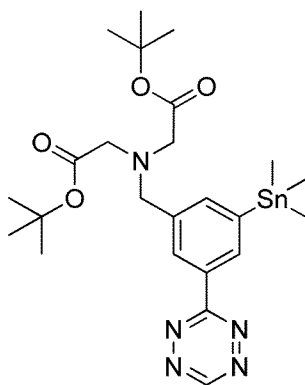
Example 100

Di-tert-butyl 2,2'-((3-iodo-5-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-156).

The compound was obtained from di-tert-butyl 2,2'-((3-cyano-5-iodobenzyl)azanediyl)diacetate (1.30 g, 2.67 mmol) following *General Procedure C*. The crude was purified using flash chromatography (heptane/EtOAc 95/5) to 0.37 g (26%) of UB-156 as red oil. *R_f* = 0.39 (n-Heptane:20%EtOAc); ¹H NMR (600 MHz, Chloroform-*d*) δ 10.22 (s, 1H), 8.84 (s, 1H), 8.55 (s, 1H), 8.10 (s, 1H), 3.99 (s, 2H), 3.44 (s, 4H), 1.46 (s, 18H); ¹³C NMR (151

MHz, Chloroform-*d*) δ 170.11, 165.31, 157.97, 142.35, 136.05, 133.37, 127.89, 95.12, 81.35, 56.66, 55.28, 28.19.

Example 101



Di-tert-butyl

2,2'-((3-(1,2,4,5-tetrazin-3-yl)-5-

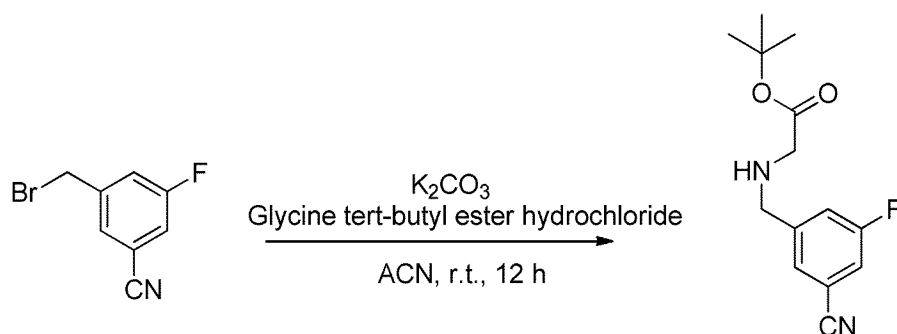
(trimethylstannyl)benzyl)azanediyl)diacetate (UB-161). The final compound was obtained from 50 mg (0.09 mmol) of the starting material, following the *General Procedure D.2*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield 0.025 g (47%) of UB-161 as a purple oil. *R_f* = 0.37 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.20 (s, 1H), 8.63 (d, *J* = 1.8 Hz, 1H), 8.55 (s, 1H), 7.97 – 7.73 (m, 1H), 4.08 (s, 2H), 3.50 (s, 4H), 1.47 (s, 18H), 0.37 (s, 9H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 170.12, 166.84, 157.74, 144.34, 141.42, 134.63, 131.05, 129.00, 81.28, 57.37, 55.09, 28.19, -9.27; HPLC-MS [M+H]⁺ *m/z* calc. for [C₂₄H₃₈N₅SnO₄]⁺: 580.19; Found: 580.22.

The synthesis of UB-108 and UB-137 using UB-161 is shown in Figure 6. Figure 7a displays the NMR chromatogram.

Example 102 of UB-108:

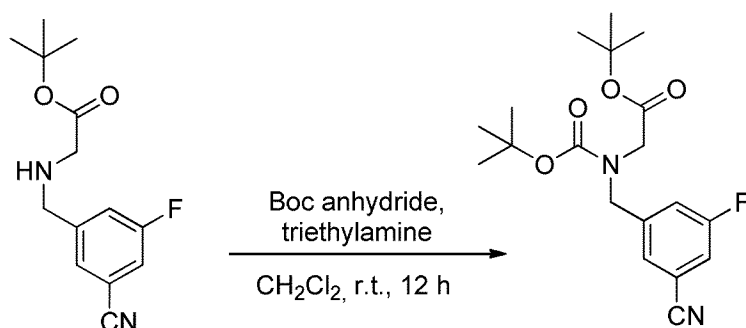
The radiolabelling of UB-108 was provided as described Example 128, 129, 130, 131 and 132 and as shown in Figure 1 and the final structure is also shown in Figure 4. The HPLC test of UB108 was performed as described in Example 135, and the HPLC chromatogram is shown in Figure 9.

Example 103



Tert-butyl 2-((3-cyano-5-fluorobenzyl)amino)acetate (UB-127). To a solution of 3-(bromomethyl)-5-fluorobenzonitrile (3.34 g, 15.60 mmol) in CH_3CN (40 mL) was added K_2CO_3 (10.78 g, 78.02 mmol) and glycine tert-butyl ester hydrochloride (7.85 g, 46.81 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography afforded 3.52 g (85%) of UB-127 as a colorless oil. $R_f = 0.23$ (n-Heptane:20%EtOAc); 1H NMR (400 MHz, Chloroform- d) δ 7.46 (d, $J = 1.5$ Hz, 1H), 7.35 (dt, $J = 9.3, 1.8$ Hz, 1H), 7.25 – 7.18 (m, 1H), 3.83 (s, 2H), 3.28 (s, 2H), 1.92 (s, 1H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, Chloroform- d) δ 171.35, 162.36 (d, $J = 250.1$ Hz), 144.68 (d, $J = 7.4$ Hz), 127.53 (d, $J = 3.2$ Hz), 119.97 (d, $J = 21.3$ Hz), 117.66 (d, $J = 24.8$ Hz), 117.63 (d, $J = 3.3$ Hz), 113.66 (d, $J = 9.7$ Hz), 81.60, 51.96 (d, $J = 1.8$ Hz), 50.82, 28.12.

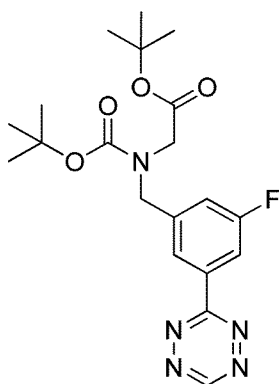
Example 104



Tert-butyl 2-((tert-butoxycarbonyl)(3-cyano-5-fluorobenzyl)amino)acetate (UB-128). To a solution of tert-butyl 2-((3-cyano-5-fluorobenzyl)amino)acetate (1.5 g, 5.67 mmol) and Et_3N (1.90 mL, 13.62 mmol) in DCM (40 mL) was added Boc_2O (1.48 g, 6.81 mmol). The reaction was stirred at room temperature for 12 h. The solution was then washed with water (50 mL) and K_2CO_3 saturated solution (50 mL), dried over anhydrous Na_2SO_4 , filtered and concentrated under reduced pressure to afford 2.1 g of the crude. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 1.84 g (89%) of UB-128 as a colorless oil (60/40 unassigned

rotamers mixture). R_f = 0.48 (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 7.42 – 7.34 (m, 1H), 7.33 – 7.25 (m, 2H), 4.54 (s, 1.2H), 4.49 (s, 0.8H), 3.89 (s, 0.8H), 3.74 (s, 1.2H), 1.67 – 1.35 (m, 18H); ^{13}C NMR (101 MHz, Chloroform- d) δ 168.51, 168.43, 162.39 (d, J = 250.6 Hz), 155.62, 155.21, 143.03 (d, J = 7.3 Hz), 142.68 (d, J = 7.3 Hz), 127.11 (d, J = 3.2 Hz), 126.85 – 126.63 (m), 119.78 (d, J = 21.6 Hz), 119.25 (d, J = 21.5 Hz), 117.99 (d, J = 24.7 Hz), 117.92 (d, J = 24.8 Hz), 117.52 – 117.42 (m), 113.90 (d, J = 9.8 Hz), 81.63 (d, J = 78.3 Hz), 81.51 (d, J = 81.3 Hz), 51.17, 50.90, 49.88, 49.52, 31.87, 29.00, 28.21, 28.03, 22.67, 14.09.

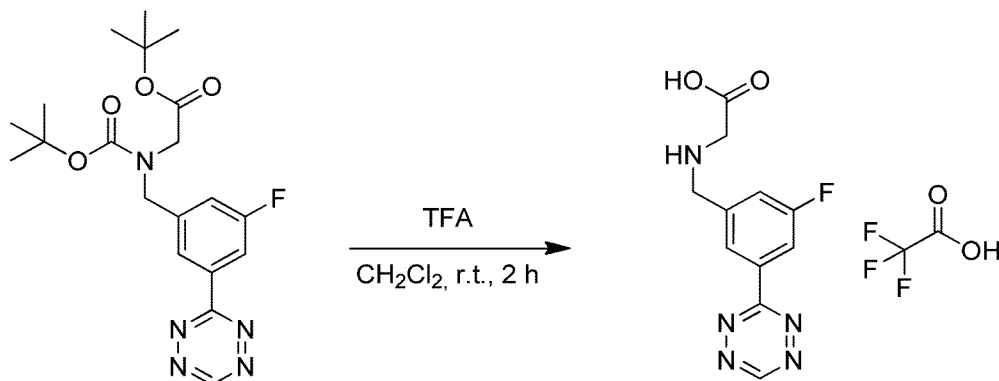
10 Example 105



Tert-butyl 2-((tert-butoxycarbonyl)(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (UB-133). The compound was obtained from tert-butyl 2-((tert-butoxycarbonyl)(3-cyano-5-

fluorobenzyl)amino)acetate (1.56 g, 4.28 mmol) following *General Procedure C*. The crude was purified using flash chromatography (heptane/EtOAc 95/5) to yield 0.45 g (25%) of UB-133 as red oil (60/40 unassigned rotamers mixture). R_f = 0.41 (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.27 (s, 1H), 8.37 – 8.30 (m, 1H), 8.27 – 8.20 (m, 1H), 7.40 – 7.28 (m, 1H), 4.67 (s, 1.2H), 4.60 (s, 0.8H), 3.93 (s, 0.8H), 3.79 (s, 1.2H), 1.53 – 1.41 (m, 18H); ^{13}C NMR (101 MHz, Chloroform- d) δ 168.64, 165.56, 163.56 (d, J = 248.0 Hz), 163.49 (d, J = 248.1 Hz), 158.02, 155.73, 155.42, 142.44 (d, J = 6.3 Hz), 142.19 (d, J = 6.9 Hz), 133.73 (d, J = 8.9 Hz), 123.05, 122.88, 119.57 (d, J = 22.0 Hz), 119.08 (d, J = 21.7 Hz), 81.43 (d, J = 82.1 Hz), 81.30 (d, J = 91.4 Hz), 51.41, 51.06, 49.60, 49.28, 28.30, 28.26, 28.04.

Example 106



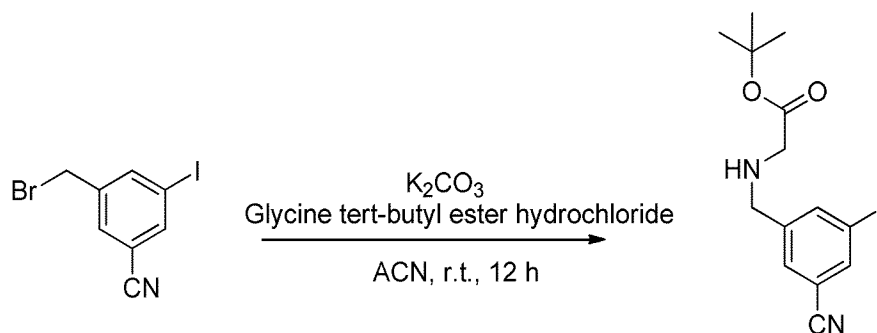
1-Carboxy-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-

trifluoroacetate (UB-137).

To a solution of tert-butyl 2-((tert-butoxycarbonyl)(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (0.30 g, 0.71 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 hours. The solvent was then removed under reduced pressure to obtain a red solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.11 g (41%) of UMB-137 as a pink oil. ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.32 (s, 1H), 8.52 (s, 1H), 8.30 (ddd, *J* = 9.4, 2.5, 1.5 Hz, 1H), 7.85 – 7.25 (m, 1H), 4.36 (s, 2H), 3.93 (s, 2H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 167.31, 165.08 (d, *J* = 3.3 Hz), 163.31 (d, *J* = 247.8 Hz), 158.31, 135.47 (d, *J* = 8.8 Hz), 134.73 (d, *J* = 7.8 Hz), 125.19 (d, *J* = 3.1 Hz), 120.87 (d, *J* = 22.9 Hz), 115.54 (d, *J* = 24.3 Hz), 49.76 (d, *J* = 1.9 Hz), 46.57; HPLC-MS [*M*+*H*]⁺ *m/z* calc. for [C₁₁H₁₁FN₅O₂]⁺: 264.09; Found: 264.07.

Figure 5 displays the whole synthesis scheme. Figure 8 displays the NMR chromatogram.

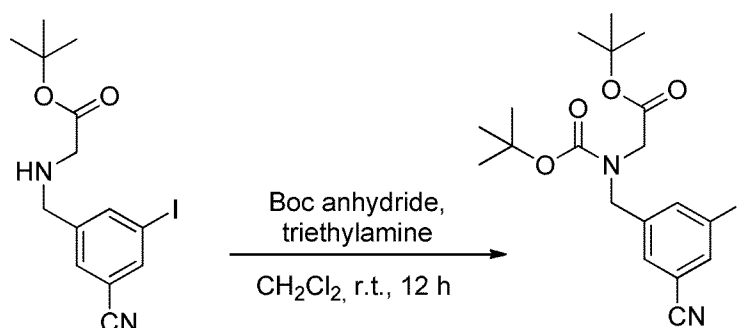
Example 107



Tert-butyl 2-((3-cyano-5-iodobenzyl)amino)acetate (UB-231). To a solution of 3-(bromomethyl)-5-iodobenzonitrile (2.00 g, 6.21 mmol) in CH₃CN (40 mL) was added K₂CO₃ (4.29 g, 31.07 mmol) and glycine tert-butyl ester hydrochloride (3.12 g, 18.63 mmol). The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo, and the

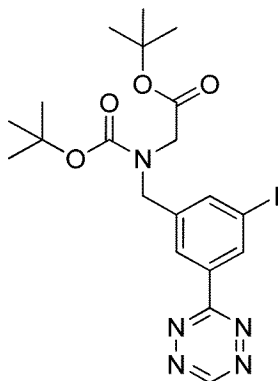
resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash chromatography afforded 1.91 g (83%) of UB-231 as a colorless oil. *R_f* = 0.23 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.89 (s, 1H), 7.79 (s, 1H), 7.56 (s, 1H), 3.72 (s, 2H), 3.21 (s, 2H), 1.91 (s, 1H), 1.41 (s, 9H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 171.24, 143.30, 141.56, 139.06, 130.89, 117.13, 114.11, 93.95, 81.68, 51.74, 50.76, 28.13.

Example 108



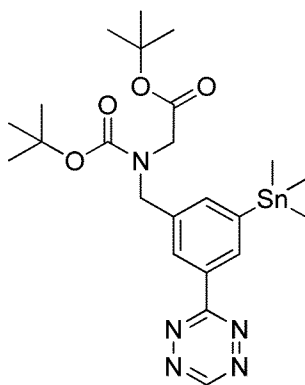
Tert-butyl 2-((tert-butoxycarbonyl(3-cyano-5-iodobenzyl)amino)acetate (UB-232). To a solution of tert-butyl 2-((3-cyano-5-iodobenzyl)amino)acetate (1.9 g, 5.10 mmol) and Et₃N (1.71 mL, 12.25 mmol) in DCM (40 mL) was added Boc₂O (1.41 g, 6.12 mmol). The reaction was stirred at room temperature for 12 hours. The solution was then washed with water (50 mL) and K₂CO₃ saturated solution (50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to afford 2.5 g of the crude. Purification by flash chromatography (80/20 Heptane/EtOAc) afforded 2.2 g (91%) of UB-232 as a colorless oil (60/40 unassigned rotamers mixture). *R_f* = 0.38 (n-Heptane:20%EtOAc); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.99 – 7.80 (m, 2H), 7.71 – 7.41 (m, 1H), 4.48 (s, 1.2H), 4.42 (s, 0.8H), 3.86 (s, 0.8H), 3.70 (s, 1.2H), 1.51 – 1.42 (m, 18H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 168.43, 155.62, 142.14, 141.49, 141.13, 140.79, 139.30, 131.33, 130.50, 130.04, 117.00, 114.29, 94.03, 82.03, 81.93, 81.30, 81.13, 50.85, 50.56, 49.78, 49.53, 28.22, 28.05.

Example 109

**Tert-butyl 2-((tert-butoxycarbonyl)(3-iodo-5-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate**

5 **(UB-235)**. The compound was obtained from tert-butyl 2-((tert-butoxycarbonyl)(3-cyano-5-iodobenzyl)amino)acetate (2.10 g, 4.44 mmol) following *General Procedure C*. The crude was purified using flash chromatography (heptane/EtOAc 95/5) to yield 0.64 g (27%) of UB-235 as red oil (60/40 unassigned rotamers mixture). R_f = 0.31 (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.18 (s, 1H), 8.84 – 8.71 (m, 1H), 8.45 – 8.36 (m, 1H), 7.87 – 7.74 (m, 1H), 4.54 (s, 1.2H), 4.47 (s, 0.8H), 3.85 (s, 0.8H), 3.69 (s, 1.2H), 1.44 – 1.36 (m, 9H), 1.38 (s, 9H); ^{13}C NMR (101 MHz, Chloroform- d) δ 168.63, 165.14, 158.04, 155.71, 155.39, 141.70, 141.46, 141.26, 140.93, 136.06, 135.93, 133.58, 133.52, 126.70, 126.48, 95.21, 81.83, 81.74, 81.07, 80.85, 51.11, 50.71, 49.47, 49.33, 28.31, 28.26, 28.07.

15 Example 110

**tert-butyl 2-((tert-butoxycarbonyl)(3-(1,2,4,5-tetrazin-3-yl)-5-(trimethylstannyl)benzyl)amino)acetate (UB-266)**

20 The final compound was obtained from 77 mg (0.15 mmol) of the starting material, following the *General Procedure D.2*. The crude was purified using flash chromatography (95/5 n-Heptane/EtOAc) to yield 0.055 g (66%) of UB-266 as a purple oil (60/40 unassigned rotamers mixture). R_f = 0.38 (n-Heptane:20%EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.21 (s, 1H), 8.74 – 8.60 (m, 1H), 8.45 – 8.37 (m, 1H), 7.70

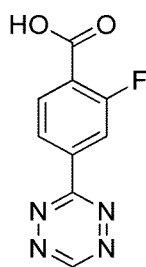
– 7.61 (m, 2H), 4.65 (s, 1.2H), 4.58 (s, 0.8H), 3.90 (s, 0.8H), 3.75 (s, 1.2H), 1.49 (s, 9H), 1.44 (s, 9H), 0.36 (s, 9H); ^{13}C NMR (101 MHz, Chloroform- d) δ 168.85, 166.70, 157.76, 155.81, 155.62, 144.69, 140.25, 139.75, 138.58, 138.33, 134.55, 131.09, 127.41, 81.62, 81.51, 80.69, 80.51, 51.60, 51.17, 49.25, 48.95, 28.37, 28.29, 28.07, -9.34; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{23}\text{H}_{36}\text{N}_5\text{SnO}_4]^+$: 566.18; Found: 566.19.

The synthesis of UB-108 and UB-137 using UB-266 is shown in Figure 6. Figure 8a displays the NMR chromatogram.

Example 111 of UB-137: See radiochemistry section and Figure 1, 6 and 12

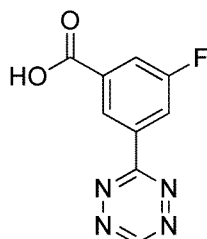
The radiolabelling of UB-134 was provided as described in the Example 128, 129, 130, 131 and 132 and as shown in Figure 1 and the final structure is also shown in Figure 4. The HPLC test of UB-137 was performed as described in Example 135, and the HPLC chromatogram is shown in Figure 10.

Example 112



2-Fluoro-4-(1,2,4,5-tetrazin-3-yl)benzoic acid (UB-12) The compound was obtained from 4-cyano-2-fluorobenzoic acid (0.66 g, 4.00 mmol) following the following *General Procedure C*. Purification by flash chromatography (98/2 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) afforded 0.32 g (36%) of UB-12 as a red solid. R_f = 0.31 (Heptane/EtOAc 30/70); ^1H NMR (600 MHz, Methanol- d_4) δ 10.44 (s, 1H), 8.50 (dd, J = 8.1, 1.6 Hz, 1H), 8.40 (dd, J = 11.5, 1.7 Hz, 1H), 8.20 (t, J = 7.7 Hz, 1H); ^{13}C NMR (151 MHz, Methanol- d_4) δ 165.11 (d, J = 3.3 Hz), 165.04 (d, J = 2.7 Hz), 162.10 (d, J = 259.0 Hz), 158.24 (d, J = 10.2 Hz), 137.97 (d, J = 8.8 Hz), 132.85 (d, J = 1.2 Hz), 123.10 (d, J = 4.0 Hz), 122.84 (d, J = 10.7 Hz), 115.83 (d, J = 25.7 Hz); MS (ESI) m/z $[\text{M} - \text{H}]^-$: 219.04

Example 113



3-Fluoro-5-(1,2,4,5-tetrazin-3-yl)benzoic acid (UB-65). The final compound was obtained from 3-Fluoro-5-cyanobenzoic acid (0.66 g, 4.00 mmol) following *General Procedure C*. The crude was purified using flash chromatography (98/2 CH₂Cl₂/MeOH) to yield 0.21 g (24%) of UB-65 as a red solid. *R*_f = 0.31 (n-Heptane:60%EtOAc); ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.32 (s, 1H), 8.95 (t, *J* = 1.5 Hz, 1H), 8.42 (ddd, *J* = 9.2, 2.6, 1.5 Hz, 1H), 7.90 (ddd, *J* = 8.8, 2.7, 1.4 Hz, 1H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 166.04, 165.07, 163.08 (d, *J* = 247.1 Hz), 158.29, 134.91 (d, *J* = 8.2 Hz), 134.49 (d, *J* = 7.3 Hz), 124.63 (d, *J* = 3.1 Hz), 119.87 (d, *J* = 23.2 Hz), 118.29 (d, *J* = 24.7 Hz); HPLC-MS [M-H]⁺ *m/z* calc. for [C₉H₄FN₄O₂]⁺: 219.03; Found: 219.07.

Example 114

Di-*tert*-butyl 2,2'-((4-cyano-2-fluorobenzyl)azanediyl)diacetate (UB-41)

The compound was obtained from 4-(bromomethyl)-3-fluorobenzonitrile (0.85 g, 3.97 mmol) following the procedure employed for example 95. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 1.5 g (90%) of the desired compound as a colorless oil. *R*_f = 0.34 (n-Heptane/EtOAc 80/20); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.83 (t, *J* = 7.5 Hz, 1H), 7.47 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.33 (dd, *J* = 9.2, 1.7 Hz, 1H), 4.03 (s, 2H), 3.45 (s, 4H), 1.48 (s, 18H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 170.21, 160.53 (d, *J* = 250.0 Hz), 132.26 (d, *J* = 14.0 Hz), 132.19 (d, *J* = 5.1 Hz), 128.20 (d, *J* = 3.8 Hz), 118.79 (d, *J* = 25.5 Hz), 117.73 (d, *J* = 2.8 Hz), 112.16 (d, *J* = 9.5 Hz), 81.34, 55.61, 50.06, 28.13.

Example 115

Di-*tert*-butyl 2,2'-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-42)

The compound was obtained from Di-*tert*-butyl 2,2'-((4-cyano-2-fluorobenzyl)azanediyl)diacetate (1.5 g, 3.96 mmol) following *General Procedure C*. Purification by flash chromatography (n-Heptane/EtOAc 85/15) afforded 0.25 g (15%) of the desired compound as a red oil. *R*_f = 0.39 (n-Heptane/EtOAc 80/20); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.16 (s, 1H), 8.34 (dd, *J* = 8.1, 1.6 Hz, 1H), 8.21 (dd, *J* = 10.6, 1.7 Hz, 1H), 7.80 (t, *J* = 7.7 Hz, 1H), 4.01 (s, 2H), 3.42 (s, 4H), 1.40 (s, 18H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 170.33, 165.69 (d, *J* = 3.0 Hz), 161.70 (d, *J* = 247.8 Hz), 157.89, 132.38, 132.31 (d, *J* = 4.4 Hz), 131.48 (d, *J* = 14.5 Hz), 124.02 (d, *J* = 3.4 Hz), 114.88 (d, *J* = 25.1 Hz), 81.21, 55.58, 50.18 (d, *J* = 2.9 Hz), 28.15.

Example 116

1-Carboxy-N-(carboxymethyl)-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (UB-44)

The compound was obtained from di-*tert*-butyl 2,2'-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.24 g, 0.55 mmol) following the procedure employed in example 97. Purification by preparative HPLC afforded 0.02 g (8%) of UB-44 as a red solid. ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.41 (s, 1H), 8.50 (dd, *J* = 8.0, 1.6 Hz, 1H), 8.38 (dd, *J* = 10.9, 1.6 Hz, 1H), 7.90 (t, *J* = 7.7 Hz, 1H), 4.49 (s, 2H), 3.96 (s, 4H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 170.22, 165.29, 162.05 (d, *J* = 248.2 Hz), 158.17, 135.18 (d, *J* = 8.2 Hz), 133.50 (d, *J* = 3.6 Hz), 125.50 (d, *J* = 13.7 Hz), 123.84 (d, *J* = 3.5 Hz), 114.61 (d, *J* = 25.2 Hz), 53.85, 51.04; HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₃H₁₃FN₅O₄]⁺: 322.09; Found: 322.12

10 Example 117

Tert-butyl 2-((4-cyano-2-fluorobenzyl)amino)acetate (UB-33)

The compound was obtained from 4-(bromomethyl)-3-fluorobenzonitrile (1.00 g, 4.67 mmol) following the procedure example 103. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 0.52 g (42%) of the desired compound as a colorless oil. *R*_f = 0.31 (n-Heptane/EtOAc 60/40); ¹H NMR (400 MHz, Chloroform-*d*) δ 7.48 (t, *J* = 7.5 Hz, 1H), 7.32 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.21 (dd, *J* = 9.4, 1.6 Hz, 1H), 3.79 (s, 2H), 3.19 (s, 2H), 1.91 (s, 1H), 1.34 (s, 9H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 171.14, 160.28 (d, *J* = 249.5 Hz), 133.12 (d, *J* = 14.8 Hz), 130.95 (d, *J* = 5.2 Hz), 128.13 (d, *J* = 3.8 Hz), 118.69 (d, *J* = 25.5 Hz), 117.51, 112.03 (d, *J* = 9.6 Hz), 81.23, 50.85, 45.89 (d, *J* = 3.1 Hz).

20

Example 118

Tert-butyl 2-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (UB-35)

Tert-butyl 2-((4-cyano-2-fluorobenzyl)amino)acetate (0.5 g, 1.89 mmol), CH₂Cl₂ (0.121 mL, 1.89 mmol), sulfur (0.12 g, 0.47 mmol) and ethanol (4.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (0.74 mL, 15.13 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH₂Cl₂ and sodium nitrite (1.30 g, 18.92 mmol) in 10 ml of H₂O were added to the mixture. Excess acetic acid (7 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (70/30 heptane/EtOAc) to yield 0.120 g (20%) of red oil. *R*_f = 0.32 (n-Heptane/EtOAc 60/40); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.44 (s, 1H), 8.51 (dd, *J* = 8.1, 1.7 Hz, 1H), 8.43 (dd, *J* = 10.8, 1.7 Hz, 1H), 7.88 (t, *J* = 7.8 Hz, 1H), 3.79 (s, 2H), 3.19 (s, 2H), 1.91 (s, 1H), 1.34 (s, 9H).

35

Example 119**1-Carboxy-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (UB-39)**

To a solution of *tert*-butyl 2-((*tert*-butoxycarbonyl)(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (0.10 g, 0.33 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.05 g (40%) of UB-39 as a red solid. ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.44 (s, 1H), 8.54 (dd, *J* = 8.1, 1.6 Hz, 1H), 8.45 (dd, *J* = 10.8, 1.6 Hz, 1H), 7.86 (t, *J* = 7.7 Hz, 1H), 4.52 (s, 2H), 4.07 (s, 2H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 167.23, 165.09 (d, *J* = 2.9 Hz), 161.74 (d, *J* = 249.3 Hz), 158.27, 136.24 (d, *J* = 8.7 Hz), 133.05 (d, *J* = 3.2 Hz), 124.10, 122.61 (d, *J* = 15.4 Hz), 114.86 (d, *J* = 24.6 Hz), 43.79 (d, *J* = 3.7 Hz); HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₁H₁₁FN₅O₂]⁺: 264.09; Found: 264.08.

Example 120**2-(4-Cyanophenyl)-N-(2-fluoroethyl)acetamide (UB-160)**

To a solution of the 2-(4-cyanophenyl)acetic acid (0.50 g, 3.10 mmol) in DCM (40 mL) was added CDI (0.65 g, 4.03 mmol) and the resulting mixture was stirred for 1 h at room temperature. Subsequently the amine (0.62 g, 6.20 mmol) was added and the reaction stirred overnight. The organic layer was washed with NaHCO₃ saturated solution (2 x 20 mL), 1 M HCl solution (2 x 20 mL) and brine (2 x 20 mL); dried over Na₂SO₄ filtered and concentrated under reduced pressure to give 0.46 g (72%) of UB-160 as a white solid. R.f.: 0.35 (Heptane/EtOAc 30/70); m.p. : 139-141 °C; ¹H-NMR (CDCl₃, 400 MHz): 3.43-3.49 (m, 1H, CH), 3.50-3.59 (m, 3H, CH + CH₂), 4.35 (t, *J* = 4.8 Hz, 1H, CH), 4.47 (t, *J* = 4.8 Hz, 1H, CH), 5.88 (br s, 1H, NH), 7.34 (d, *J* = 8.2 Hz, 1H, CH), 7.57 (d, *J* = 8.2 Hz, 1H, CH); ¹³C-NMR (CDCl₃, 100 MHz): 40.2 (*J*_{C-F} = 19.5 Hz), 43.4, 82.5 (*J*_{C-F} = 166.7 Hz), 111.3, 118.6, 130.1, 132.6, 140.0, 169.6.

Example 120a**2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-N-(2-fluoroethyl)acetamide (UB-163)**

The compound was obtained from *tert*-butyl 2-(4-Cyanophenyl)-N-(2-fluoroethyl)acetamide (0.40 g, 1.94 mmol,) following *General Procedure C*. The resulting residue was purified using flash chromatography (30/70 Heptane/EtOAc) to yield 0.15 g of a red solid. The powder was triturated in DCM and filtered to afford 0.21 g (41%) of UB-163 as a pink solid. R.f. = 0.22 (Heptane/EtOAc 40/60); ¹H-NMR (DMSO-*d*₆, 400 MHz): 3.36 (q, *J* = 5.2 Hz, 1H, CH), 3.43 (q, *J* = 5.2 Hz, 1H, CH), 3.62 (s, 2H, CH₂), 4.39 (t, *J* = 5.0 Hz, 1H, CH), 5.02 (t, *J* = 5.0

Hz, 1H, CH), 7.57 (d, J = 8.2 HZ, 2H, Ar-H), 8.39-8.49 (m, 3H, NH + Ar-H), 10.58 (s, 1H, Ar-H); ¹³C-NMR (DMSO-d₆, 100 MHz): 40.6 (J_{C-F} = 21.2 Hz), 42.6, 82.9 (J_{C-F} = 165.0 Hz), 128.1, 130.5, 130.6, 142.0, 158.5, 165.9, 170.3.

5 Example 120b

5-((4-cyano-2-fluorophenyl)amino)-5-oxopentanoic acid (UB-34)

To a solution of 3-fluoro-4-aminobenzonitrile (1.0 g, 7.34 mmol) in anhydrous THF (20.0 mL) was added under nitrogen glutaric anhydride (0.84 g, 7.34 mmol). The reaction was refluxed 24 h. The solvent was removed under reduced pressure and the crude purified by flash chromatography (95/5 DCM/MeOH) to give 1.81 g of a yellow solid. Recrystallization from EtOAc afforded 1.65 g (90%) of UB-34 as a white solid. m.p.: 140-142 °C; ¹H-NMR (DMSO-d₆, 400 MHz): 1.81 (quint, J = 7.4 Hz, 2H, CH₂), 2.28 (t, J = 7.4 Hz, 2H, CH₂), 2.49 (t, J = 7.4 Hz, 2H, CH₂), 7.65 (dd, J = 1.2, 8.6 Hz, 1H, Ar-H), 7.88 (d, J = 1.9, 11.0, Hz, 1H, Ar-H), 8.27 (pseudo t, J = 8.2 Hz, 1H, Ar-H), 10.09 (br s, 1H, NH), 12.07 (br s, 1H, COOH); ¹³C-NMR (DMSO-d₆, 100 MHz): 20.7, 33.3, 35.5, 106.3 (J_{C-F} = 9.4 Hz), 118.3, 119.8 (J_{C-F} = 23.4 Hz), 123.6 (J_{C-F} = 2.9 Hz), 129.8 (J_{C-F} = 3.5 Hz), 132.0 (J_{C-F} = 11.3 Hz), 152.2 (J_{C-F} = 247.5 Hz), 172.4, 174.5.

Example 120c

5-((4-fluoro-4-(1,2,4,5-tetrazin-3-yl)phenyl)amino)-5-oxopentanoic acid (UB-37)

5-((4-Cyano-2-fluorophenyl)amino)-5-oxopentanoic acid (0.80 g, 3.20 mmol), CH₂Cl₂ (3.19 mmol, 0.204 mL), sulfur (0.204 g, 0.80 mmol) and ethanol (5.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (1.24 mL, 25.57 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH₂Cl₂ and sodium nitrite (2.20 g, 31.97 mmol) in 40 ml of H₂O were added to the mixture. Excess acetic acid (14 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (95/5 DCM/MeOH) to yield 0.25 g of a red solid. The NMR shows some impurity so the compound was crystallized from EtOH to 0.210 (21%) of UB-37 as a red solid. R.f. = 0.27 (DCM/MeOH 95/5); mp = 205-207 °C; ¹H-NMR (DMSO-d₆, 400 MHz): 1.83 (quint, J = 7.3 Hz, 2H, CH₂), 2.31 (t, J = 7.4 Hz, 2H, CH₂), 2.53 (t, J = 7.4 Hz, 2H, CH₂), 8.24-8.39 (m, 3H, Ar-H), 10.07 (br s, 1H, NH), 10.58 (s, 1H, Ar-H) 12.08 (br s, 1H, COOH); ¹³C-NMR (DMSO-d₆, 100 MHz): 20.8, 33.4, 35.5, 114.8 (J_{C-F} = 22.2 Hz), 123.9 (J_{C-F} = 2.4 Hz), 124.7 (J_{C-F} = 3.2

Hz), 128.1 (J_{C-F} = 7.7 Hz), 131.3 (J_{C-F} = 11.3 Hz), 153.4 (J_{C-F} = 245.8 Hz), 158.4, 164.9 (J_{C-F} = 3.0 Hz), 174.5

Example 120d

5 Tert-butyl 4-cyano-2-fluorobenzylcarbamate (UB-32)

4-(Aminomethyl)-3-fluorobenzonitrile hydrochloride (1.5 g, 8.04 mmol) and triethylamine (2.35 mL, 16.87 mmol) were dissolved in anhydrous DCM (40 mL) at 0 ° C. To this stirred solution was added di-tert-butyl dicarbonate (2.10 g, 9.64 mmol), and the reaction allowed to warm to room temperature and stirred for 16 hours. The reaction mixture was evaporated in vacuo, and the residue was re-dissolved in diethyl ether (50 mL), which was washed successively with 0.5 M aq. HCl (2 x 25 mL), saturated NaHCO₃ (2 x 25 mL) and brine (25 mL). The organic layer was dried with MgSO₄, filtered and evaporated in vacuo to give an off-white solid. The residue was purified by flash column chromatography (Heptane/EtOAc = 85/15) to afford 1.51 g (75%) of UB-32 as an orange solid. m.p.: 51-53 °C; ¹H-NMR (CDCl₃, 400 MHz): 1.47 (s, 9H, C(CH₃)₃), 4.42 (d, J = 4.4 Hz, 2H, CH₂), 4.99 (br s, 1H, NH), 7.36 (dd, J = 1.4, 9.4 Hz, 1H, Ar-H), 7.42-7.56 (m, 2H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.2, 30.0, 79.8, 112.1 (J_{C-F} = 9.5 Hz), 117.4 (J_{C-F} = 2.9 Hz), 118.7 (J_{C-F} = 25.0 Hz), 128.2 (J_{C-F} = 3.9 Hz), 130.1 (J_{C-F} = 5.0 Hz), 132.6 (J_{C-F} = 15.5 Hz), 156.0, 159.9 (J_{C-F} = 249.8 Hz).

20 Example 120e

Tert-butyl 2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzylcarbamate (UB-35)

Tert-butyl 4-cyano-2-fluorobenzylcarbamate (1.5 g, 5.99 mmol), CH₂Cl₂ (5.99 mmol, 0.38 mL), sulfur (0.38 g, 1.49 mmol) and ethanol (7.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (2.33 mL, 47.94 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 12 ml of CH₂Cl₂ and sodium nitrite (4.13 g, 59.93 mmol) in 30 ml of H₂O were added to the mixture. Excess acetic acid (25 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (90/10 Heptane/EtOAc) and recrystallized from Heptane to yield 0.39 g (21%) of UB-35 as a red solid. R.f = 0.38 (Heptane/EtOAc 80/20); ¹H-NMR (CDCl₃, 400 MHz): 1.48 (s, 9H, C(CH₃)₃), 4.49 (d, J = 6.3 Hz, 2H, CH₂), 5.11 (br s, 1H, NH), 7.60 (pseudo t, J = 7.7 Hz, 1H, Ar-H), 8.30 (dd, J = 1.7, 10.8 Hz, 1H, Ar-H), 8.41 (dd, J = 1.6, 8.0 Hz, 1H, Ar-H), 10.25 (s, 1H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.4, 38.6, 80.0, 115.0 (J_{C-F} = 24.5 Hz), 124.1, 130.5, 131.6 (J_{C-F} = 15.0 Hz), 132.6 (J_{C-F} = 8.5 Hz), 155.8, 157.9, 161.2 (J_{C-F} = 247.6 Hz), 165.5.

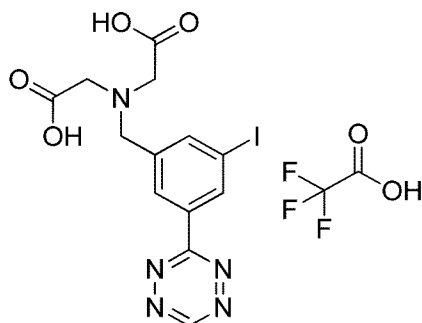
Example 120f**(2-Fluoro-4-(1,2,4,5-tetrazin-3-yl)phenyl)methanamine (UB-38)**

To a solution of tert-butyl 2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzylcarbamate (0.300 g, 0.98 mmol) in DCM (20 mL) was added a solution of HCl in diethyl ether (1.0 M, 20.0 mL). The mixture was stirred at room temperature for 72 h. The reaction was then concentrated under reduced pressure to give 0.23 g (97%) of UB-38 as a pink solid. ¹H-NMR (MeOD, 400 MHz): 2.17 (s, 2H, CH₂), 7.74 (pseudo t, J = 7.8 Hz, 1H, Ar-H), 8.33 (dd, J = 1.6, 10.9 Hz, 1H, Ar-H), 8.43 (dd, J = 1.6, 8.0 Hz, 1H, Ar-H), 10.34 (s, 1H, Ar-H); ¹³C-NMR (MeOD, 100 MHz): 36.4 (J_{C-F} = 4.2 Hz), 114.7 (J_{C-F} = 24.5 Hz), 124.1 (J_{C-F} = 3.7 Hz), 124.8 (J_{C-F} = 15.3 Hz), 132.0 (J_{C-F} = 3.5 Hz), 135.6 (J_{C-F} = 8.4 Hz), 158.3, 161.4 (J_{C-F} = 248.3 Hz), 165.1.

Example 120g**5-((2-Fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-5-oxopentanoic acid (UB-40)**

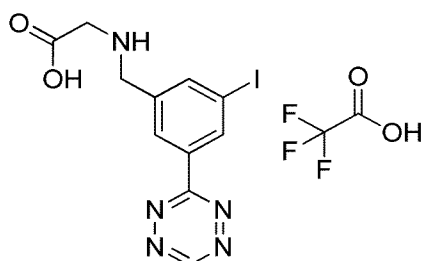
To a solution of UB-38 (0.20 g, 0.83 mmol), and Et₃N (0.34 mL, 2.48 mmol) in THF (20 mL) was added glutaric anhydride (0.14 g, 1.24 mmol). The reaction was stirred at 50 °C for 2 h. The solvent was then removed under reduced pressure and the residue solubilized in water (20 mL). The pH was adjusted to 2 with 1 M HCl and the reaction mixture was extracted with EtOAc (3 x 20 mL). The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure to give 0.300 g of crude material (NMR confirmed full conversion). The resulting residue was purified using flash chromatography (95/5 DCM/MeOH) and crystallized from EtOH to give 0.21 g of UB-40 as a red solid. R.f. = 0.21 (DCM/MeOH 95/5); m.p. = 147-149 °C; ¹H-NMR (CD₃OD, 400 MHz): 1.95 (quint, J = 7.4 Hz, 2H, CH₂), 2.37 (t, J = 7.4 Hz, 4H, 2 x CH₂), 4.52 (s, 2H, CH₂), 7.63 (pseudo t, J = 7.8 Hz, 1H, Ar-H), 8.29 (dd, J = 1.7, 11.0 Hz, 1H, Ar-H), 8.41 (dd, J = 1.7, 8.1 Hz, 1H, Ar-H), 10.37 (s, 1H, Ar-H); ¹³C-NMR (CD₃OD, 100 MHz): 20.8, 32.7, 34.5, 36.4 (J_{C-F} = 4.5 Hz), 114.1 (J_{C-F} = 24.6 Hz), 123.6 (J_{C-F} = 3.4 Hz), 130.3 (J_{C-F} = 4.5 Hz), 130.7 (J_{C-F} = 15.1 Hz), 158.0, 161.1 (J_{C-F} = 246.6 Hz), 165.4, 174.1, 175.4.

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Example 121

5 **1-carboxy-N-(carboxymethyl)-N-(3-iodo-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (UB-164)**

To a solution of Di-tert-butyl 2,2'-((3-iodo-5-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-156) (0.05 g, 0.15 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.05 g (62%) of UB-164 as a pink solid. ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.30 (s, 1H), 8.87 (s, 1H), 8.64 (s, 1H), 8.14 (s, 1H), 4.45 (s, 2H), 4.01 (s, 4H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 168.19, 164.71, 158.23, 143.71, 137.65, 134.59, 134.01, 129.55, 94.51, 57.76, 53.56.

Example 122

1-carboxy-N-(3-iodo-5-(1,2,4,5-tetrazin-3-yl)benzyl)N-(tert-butoxycarbonyl)methanaminium 2,2,2-trifluoroacetate (UB-240)

To a solution of tert-butyl 2-((tert-butoxycarbonyl)(3-iodo-5-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (UB-235) (0.07 g, 0.13 mmol) in DCM (6 mL) was added trifluoroacetic acid (6 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.04 g (65%) of UMB-240 as a pink oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 10.42 (d, *J* = 1.3 Hz, 1H), 9.01 (d, *J* = 1.7 Hz, 1H), 8.76

(d, $J = 1.7$ Hz, 1H), 8.22 (d, $J = 1.7$ Hz, 1H), 4.40 (s, 2H), 4.02 (s, 2H); ^{13}C NMR (101 MHz, Chloroform- d) δ 171.21, 168.65, 162.21, 146.63, 141.53, 138.77, 138.15, 132.44, 98.49, 53.50, 50.50.

5 Example 123

Di-tert-butyl 2,2'-((4-cyano-2-iodobenzyl)azanediyl)diacetate (UB-45)

To a solution of 4-cyano-2-iodobenzylbromide (2.0 g, 6.21 mmol) in CH_3CN (50 mL) was added K_2CO_3 (1.28 g, 9.32 mmol) and the corresponding amine (1.83 g, 7.45 mmol). The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 50 mL), washed with brine (30 mL), dried over MgSO_4 , filtered and concentrated in vacuo to give 3.01 g (99%) of UB-45 as a yellow oil. R.f. = 0.32 (80/20 Heptane/EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 7.99 (d, $J = 1.6$ Hz, 1H), 7.79 (d, $J = 8.0$ Hz, 1H), 7.56 (dd, $J = 8.0, 1.7$ Hz, 1H), 3.90 (s, 2H), 3.38 (s, 4H), 1.39 (s, 18H); ^{13}C NMR (101 MHz, Chloroform- d) δ 170.28, 147.15, 142.08, 131.71, 130.39, 117.24, 112.49, 98.69, 62.50, 55.72, 28.18.

Example 123a

Di-tert-butyl 2,2'-((2-iodo-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-46)

Di-tert-butyl 2,2'-((4-cyano-2-iodobenzyl)azanediyl)diacetate (3.0 g, 6.17 mmol), CH_2Cl_2 (0.39 mL, 6.17 mmol), sulfur (0.39 g, 1.54 mmol) and ethanol (6.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (2.40 mL, 49.34 mmol) was added slowly with stirring afterwards. The vessel was sealed, and the reaction mixture was heated to 50 °C for 24 hours. Then 12 ml of CH_2Cl_2 and sodium nitrite (4.25 g, 61.68 mmol) in 60 ml of H_2O were added to the mixture. Excess acetic acid (30 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was basified with NaHCO_3 saturated solution and extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO_4), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (90/10 heptane/EtOAc) and recrystallized from heptane to yield 0.71 g (21%) of UB-46 as a red solid. R.f. = 0.33 (80/20 Heptane/EtOAc); ^1H NMR (400 MHz, Chloroform- d) δ 10.16 (s, 1H), 9.01 (d, $J = 1.8$ Hz, 1H), 8.53 (dd, $J = 8.1, 1.8$ Hz, 1H), 7.88 (d, $J = 8.1$ Hz, 1H), 4.01 (s, 2H), 3.45 (s, 4H), 1.41 (s, 18H); ^{13}C NMR (101 MHz, Chloroform- d) δ 170.21, 165.29, 157.90, 146.48, 138.84, 132.03, 130.94, 127.96, 99.98, 81.40, 62.44, 55.58, 28.21.

Example 123b**1-Carboxy-N-(carboxymethyl)-N-(2-iodo-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (UB-50)**

To a solution of di-tert-butyl 2,2'-((2-iodo-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.14 g, 0.26 mmol) in DCM (5 mL) was added trifluoroacetic acid (4 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.11 g (78%) of UMB-50 as a pink solid. ¹H NMR (400 MHz, Methanol-*d*₄) δ 10.39 (s, 1H), 9.09 (d, *J* = 1.7 Hz, 1H), 8.64 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.96 (d, *J* = 8.1 Hz, 1H), 4.43 (s, 2H), 3.92 (s, 4H); ¹³C NMR (101 MHz, Methanol-*d*₄) δ 170.76, 164.89, 158.11, 142.21, 138.85, 133.82, 131.71, 127.60, 100.50, 62.38, 53.99

Example 123c**Tert-butyl 2-((4-cyano-2-iodobenzyl)amino)acetate (UB-120)**

To a solution of 2-iodo-4-cyano-benzylbromide (1.50 g, 4.66 mmol) in CH₃CN (40 mL) was added K₂CO₃ (3.22 g, 23.29 mmol) and tert-butylglycinate HCl (2.34 g, 13.98 mmol). The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash chromatography (80/20 Heptane/EtOAc) afforded 1.45 g (84%) of UB-120 as a colorless oil. R.f = 0.24 (heptane/EtOAc 80/20); ¹H NMR (400 MHz, Chloroform-*d*) δ 8.08 (d, *J* = 1.6 Hz, 1H), 7.67 – 7.53 (m, 2H), 3.85 (s, 2H), 3.33 (s, 2H), 2.02 (s, 1H), 1.48 (s, 9H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 171.29, 147.65, 142.16, 131.76, 129.25, 117.10, 112.45, 98.90, 81.53, 57.47, 51.03, 28.13.

Example 123d**Tert-butyl 2-((tert-butoxycarbonyl)(4-cyano-2-iodobenzyl)amino)acetate (UB-123)**

To a solution of UB-120 (1.4 g, 3.76 mmol) and Et₃N (1.26 mL, 9.03 mmol) in DCM (40 mL) was added Boc₂O (0.91 g, 4.51 mmol). The reaction was stirred at room temperature for 12 h. The solution was then washed with water (50 mL) and K₂CO₃ saturated solution (50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to afford 2.1 g of the crude. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 1.42 g (80%) of UB-123 as a white solid. R.f. = 0.25 (Heptane/EtOAc 80/20); ¹H NMR (400 MHz, Chloroform-*d*) δ 8.13 – 8.06 (m, 1H), 7.72 – 7.61 (m, 1H), 7.52 – 7.42 (m, 1H), 4.58 (s, 1.2H), 4.50 (s, 0.8H), 3.92 (s, 0.8H), 3.79 (s, 1.2H), 1.52 – 1.43 (m, 11H), 1.41 (s, 4H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 168.53, 168.47, 155.60, 155.37, 145.86, 145.55, 142.35, 142.30, 131.88,

131.74, 128.58, 127.98, 117.02, 112.71, 97.71, 97.22, 81.90, 81.59 (d, $J = 83.6$ Hz), 81.13, 57.31, 56.91, 50.37, 49.81, 28.21, 28.09.

Example 123e

5 **Tert-butyl 2-((tert-butoxycarbonyl)(2-iodo-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (UB-126)**

Tert-butyl 2-((tert-butoxycarbonyl)(4-cyano-2-iodobenzyl)amino)acetate (1.30 g, 2.75 mmol), CH_2Cl_2 (0.176 mL, 2.75 mmol), sulfur (0.176 g, 2.75 mmol) and ethanol (3.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (1.10 mL, 22.02 mmol)
 10 was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH_2Cl_2 and sodium nitrite (1.89 g, 27.52 mmol) in 30 ml of H_2O were added to the mixture. Excess acetic acid (13 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was basified with NaHCO_3 saturated solution and extracted with dichloromethane. The organic phase was dried
 15 over anhydrous magnesium sulfate (MgSO_4), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (85/15 heptane/EtoAc) to yield 0.22 g (15%) of UB-126 as red solid. R.f. = 0.24 (Heptane/EtoAc 80/20); ^1H NMR (400 MHz, Chloroform- d) δ 10.25 (s, 1H), 9.13 – 9.03 (m, 1H), 8.70 – 8.57 (m, 1H), 7.60 – 7.51 (m, 1H), 4.67 (s, 1.2H), 4.59 (s, 0.8H), 3.97 (s, 0.8H), 3.83 (s, 1.2H), 1.57 –
 20 1.37 (m, 18H); ^{13}C NMR (101 MHz, Chloroform- d) δ 168.67, 168.62, 165.12, 157.95, 155.67, 155.56, 145.45, 145.21, 132.09, 129.07, 128.46, 128.05, 127.92, 98.89, 98.39, 81.87, 81.77, 80.98, 80.90, 57.05, 56.57, 50.08, 49.60, 28.26, 28.11.

Example 123f

25 **1-Carboxy-N-(2-iodo-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (UB-138)**

To a solution of *tert*-butyl 2-((tert-butoxycarbonyl)(2-iodo-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (0.10 g, 0.19 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed
 30 under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.04 g (43%) of UMB-138 as a pink solid. ^1H NMR (400 MHz, Methanol- d_4) δ 10.32 (s, 1H), 9.06 (d, $J = 1.7$ Hz, 1H), 8.60 (dd, $J = 8.1, 1.8$ Hz, 1H), 7.73 (d, $J = 8.1$ Hz, 1H), 4.49 (s, 2H), 4.00 (s, 2H); ^{13}C NMR (101 MHz, Methanol- d_4) δ 167.23, 164.65, 158.25, 139.13, 138.52, 134.84, 130.89, 128.04, 100.35, 54.74, 46.93.

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Example 124**Di-tert-butyl 2,2'-((4-cyanobenzyl)azanediyl)diacetate (UB-14)**

To a suspension of 4-cyanobenzylamine hydrochloride (0.67 g, 4.00 mmol) in DCM (20 mL) was added triethylamine (2.78 mL, 20.00 mmol) and tert-butylbromoacetate (2.93 mL, 20.00 mL). The reaction was stirred at room temperature for 12 h. The solution was then washed with water (2 x 20 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography (24 g column, 100% DCM to 98/2 DCM/MeOH) to give 0.85 g (59%) of UB-14 as a colorless oil. ¹H-NMR (CDCl₃, 400 MHz): 1.39 (br s, 18H, 2 x C(CH₃)₃), 3.14 (s, 4H, 2 x CH₂), 3.90 (s, 2H, CH₂), 7.49 (d, J = 8.2 Hz, 2H, Ar-H), 7.54 (d, J = 8.2 Hz, 2H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.2, 55.2, 57.2, 81.3, 111.1, 119.0, 129.5, 132.2, 144.7, 170.2.

Example 124a**Di-tert-butyl 2,2'-((4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-16)**

Di-tert-butyl 2,2'-((4-cyanobenzyl)azanediyl)diacetate (0.85 g, 2.36 mmol), CH₂Cl₂ (0.151 mL, 2.36 mmol), sulfur (0.151 g, 0.58 mmol) and ethanol (4.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (0.92 mL, 18.86 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH₂Cl₂ and sodium nitrite (1.63 g, 23.58 mmol) in 40 ml of H₂O were added to the mixture. Excess acetic acid (14 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was basified with NaHCO₃ saturated solution and extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (85/15 heptane/EtoAc) to yield 0.14 g (14%) of UB-16 as a red oil. ¹H-NMR (CDCl₃, 400 MHz): 1.40 (s, 18H, 3 x C(CH₃)₃), 3.39 (s, 4H, 2 x CH₂), 3.96 (s, 2H, CH₂), 7.59 (d, J = 8.4 Hz, 2H, Ar-H), 8.50 (d, J = 8.4 Hz, 1H, Ar-H), 10.13 (s, 1H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.2, 55.3, 57.3, 81.1, 128.3, 129.8, 130.5, 157.7, 166.4, 170.4.

Example 124b**N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxy-N-(carboxymethyl)methanaminium 2,2,2-trifluoroacetate (UB-19)**

To a solution of di-tert-butyl 2,2'-((4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.13 g, 0.31 mmol) in DCM (5 mL) was added TFA (3 mL). The reaction was stirred at room temperature for 1 h. The solvent was then evaporated under reduced pressure to give 0.14 g of crude. NMR confirmed full conversion. Purification by preparative HPLC to give 0.035 g

(26%) of UB-19 as a red solid. m.p.: 100-102 °C; ¹H-NMR (MeOD, 400 MHz): 3.89 (s, 4H, 2 x CH₂), 4.36 (s, 2H, CH₂), 7.68 (d, J = 8.4 Hz, 2H, Ar-H), 8.55 (d, J = 8.4 Hz, 2H, Ar-H), 10.27 (s, 1H, Ar-H); ¹³C-NMR (MeOD, 100 MHz): 53.6, 58.0, 128.2, 131.2, 132.9, 137.5, 158.0, 166.0, 169.5.

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Example 124c**Tert-butyl 2-((4-cyanobenzyl)amino)acetate (UB-15)**

To a suspension of 4-cyanobenzylamine hydrochloride (0.67 g, 4.00 mmol) in DCM (20 mL) was added triethylamine (1.22 mL, 8.80 mmol) and tert-butylbromoacetate (0.64 mL, 20.00 mL). The reaction was stirred at room temperature for 12 h. The solution was then washed with water (2 x 20 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography (24 g column, 100% DCM to 98/2 DCM/MeOH) to give 0.42 g (43%) of UB-15 as a colorless oil. ¹H-NMR (CDCl₃, 400 MHz): 1.49 (br s, 9H, C(CH₃)₃), 1.95 (br s, 1H, NH), 3.34 (s, 2H, CH₂), 3.90 (s, 2H, CH₂), 7.48 (d, J = 8.0 Hz, 2H, Ar-H), 7.64 (d, J = 8.0 Hz, 2H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.1, 50.4, 52.5, 81.8, 111.2, 118.8, 129.0, 132.3, 144.5, 170.0.

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Example 124d**N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxymethanaminium 2,2,2-trifluoroacetate (UB-28)**

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Tert-butyl 2-((4-cyanobenzyl)amino)acetate (0.73 g, 2.96 mmol), CH₂Cl₂ (0.190 mL, 2.96 mmol), sulfur (0.19 g, 0.74 mmol) and ethanol (4.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (1.15 mL, 23.71 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH₂Cl₂ and sodium nitrite (2.05 g, 29.64 mmol) in 40 ml of H₂O were added to the mixture. Excess acetic acid (14 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (80/20 heptane/EtoAc) to yield 0.170 g of tert-butyl 2-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate as a red oil. The compound (0.10 g, 0.33 mmol) was solubilized in DCM (5 mL) and to this mixture was added TFA (3 mL). The reaction was stirred at room temperature for 1 h. The solvent was then evaporated under reduced pressure to give 0.14 g of crude. NMR confirmed full conversion. The compound was crystallized from MeOH to give 0.030 g (37%) of UMB-28 as a pink solid. m.p.: 186-188 °C; ¹H-NMR (MeOD, 400 MHz): 4.13 (s, 1.25H, CH₂), 4.89 (s, 0.75H, CH₂), 4.92 (s, 0.75H, CH₂), 5.49 (s, 1.25H, CH₂), 7.33 (d,

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J = 8.4 Hz, 0.75H, Ar-H), 7.55 (d, J = 8.4 Hz, 1.25H, Ar-H), 8.43 (d, J = 8.4 Hz, 0.75H, Ar-H), 8.52 (d, J = 8.4 Hz, 1.25H, Ar-H), 10.22 (s, 0.33H, Ar-H), 10.25 (s, 0.67H, Ar-H); ¹³C-NMR (MeOD, 100 MHz): 45.0, 52.1, 55.7, 128.0, 128.2, 128.9, 129.1, 131.5, 132.3, 139.3, 139.8, 157.89, 157.94, 166.0, 167.3, 169.9.

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Example 124e**Di-tert-butyl 2,2'-((3-cyanobenzyl)azanediyl)diacetate (UB-74)**

To a solution of 3-bromomethylbenzonitrile (1.00 g, 5.10 mmol) in CH₃CN (30 mL) was added K₂CO₃ (1.06 g, 7.65 mmol) and the corresponding amine (1.50 g, 6.12 mmol). The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 1.62 g (88%) of UB-74 as a colorless oil. ¹H-NMR (CDCl₃, 400 MHz): 1.46 (s, 18H, 2 x C(CH₃)₃), 3.39 (s, 4H, 2 x CH₂), 3.92 (s, 2H, CH₂), 7.42 (t, J = 7.7 Hz, 1H, Ar-H), 7.51-7.58 (m, 1H, Ar-H), 7.64-7.70 (m, 1H, Ar-H), 7.74 (s, 1H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.1, 55.2, 56.7, 81.2, 112.4, 118.8, 129.1, 131.0, 132.3, 133.3, 140.6, 170.2.

Example 124f**Di-tert-butyl 2,2'-((3-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (UB-77)**

Di-tert-butyl 2,2'-((3-cyanobenzyl)azanediyl)diacetate (1.60 g, 4.43 mmol), CH₂Cl₂ (0.284 mL, 4.43 mmol), sulfur (0.284 g, 1.11 mmol) and ethanol (4.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (1.73 mL, 35.51 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH₂Cl₂ and sodium nitrite (3.06 g, 44.39 mmol) in 40 ml of H₂O were added to the mixture. Excess acetic acid (16 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was basified with NaHCO₃ saturated solution and extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (85/15 heptane/EtOAc) to yield 0.37 g (20%) of UB-77 as a red oil. R.f. = 0.41 (Heptane/EtOAc 80/20); ¹H-NMR (CDCl₃, 400 MHz): 1.46 (s, 9H, 2 x (C(CH₃)₃), 3.45 (s, 2H, 2 x CH₂), 4.02 (s, 2H, CH₂), 7.56 (t, J = 7.7 Hz, 1H, Ar-H), 7.77 (d, J = 7.7 Hz, 1H, Ar-H), 7.85 (d, J = 7.7 Hz, 1H, Ar-H), 8.59 (s, 1H, Ar-H); 10.20 (s, 1H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.2, 55.3, 57.2, 81.1, 127.3, 128.7, 129.4, 131.6, 133.9, 140.3, 157.8, 166.5, 170.4.

Example 124g**N-(3-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxy-N-(carboxymethyl)methanaminium 2,2,2-trifluoroacetate (UB-80)**

To a solution of di-tert-butyl 2,2'-((3-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.15 g, 0.36 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.08 g (53%) of UMB-80 as a pink solid. ¹H-NMR (MeOD, 400 MHz): 4.21 (s, 4H, 2 x CH₂), 4.71 (s, 2H, CH₂), 7.77 (t, J = 7.4 Hz, 1H, Ar-H), 7.88 (d, J = 7.4 Hz, 1H, Ar-H), 8.60-8.73 (m, 1H, Ar-H), 8.8 (s, 1H, Ar-H), 10.40 (s, 1H, Ar-H); ¹³C-NMR (MeOD, 100 MHz): 53.3, 58.5, 129.3, 130.0, 130.6, 131.1, 133.1, 135.4, 158.1, 165.8, 167.6.

Example 124h**Tert-butyl 2-((3-cyanobenzyl)amino)acetate (UB-81)**

To a solution of 3-cyano-benzylbromide (1.80 g, 9.18 mmol) in CH₃CN (40 mL) was added K₂CO₃ (6.34 g, 45.91 mmol) and tert-butylglycinate HCl (4.62 g, 27.54 mmol). The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash chromatography afforded 1.32 g (58%) of UB-81 as a colorless oil. ¹H-NMR (CDCl₃, 400 MHz): 1.47 (s, 9H, C(CH₃)₃), 3.29 (s, 2H, CH₂), 3.83 (s, 2H, CH₂), 7.43 (t, J = 7.7 Hz, 1H, Ar-H), 7.54 (d, J = 7.7 Hz, 1H, Ar-H), 7.59 (d, J = 7.7 Hz, 1H, Ar-H), 7.66 (s, 1H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.1, 50.8, 52.4, 81.4, 112.5, 118.8, 129.2, 130.8, 131.7, 132.6, 141.4, 171.4.

Example 124i**Tert-butyl 2-((tert-butoxycarbonyl)(3-cyanobenzyl)amino)acetate (UB-85)**

To a solution of tert-butyl 2-((3-cyanobenzyl)amino)acetate (1.16 g, 4.70 mmol) and Et₃N (1.57 mL, 11.30 mmol) in DCM (50 mL) was added Boc₂O (1.23 g, 5.65 mmol). The reaction was stirred at room temperature for 12 h. The solution was then washed with water (50 mL) and K₂CO₃ saturated solution (50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to afford 1.60 g (98%) of UB-85 as a yellow oil. ¹H-NMR (CDCl₃, 400 MHz): 1.37-1.49 (m, 18H, 2 x C(CH₃)₃), 3.70 (s, 1.25H, CH₂), 3.85 (s, 0.75H, CH₂), 4.48 (s, 0.75H, CH₂), 4.53 (s, 1.25H, CH₂), 7.37-7.60 (m, 4H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.0, 28.2, 49.3, 49.5, 50.9, 51.3, 80.8, 80.9, 81.7, 81.8, 112.6, 118.7, 129.3, 130.8, 131.0, 131.2, 131.8, 132.4, 155.3, 155.6, 168.6.

Example 124l**Tert-butyl 2-((3-(1,2,4,5-tetrazin-3-yl)benzyl)(tert-butoxycarbonyl)amino)acetate (UB-88)**

Tert-butyl 2-((tert-butoxycarbonyl)(3-cyanobenzyl)amino)acetate (1.45 g, 4.18 mmol), CH₂Cl₂ (0.268 mL, 4.18 mmol), sulfur (0.268 g, 1.04 mmol) and ethanol (4.0 mL) were mixed together in a 20 ml microwave reaction tube. Hydrazine monohydrate (1.63 mL, 33.48 mmol) was added slowly with stirring afterwards. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. Then 3 ml of CH₂Cl₂ and sodium nitrite (2.88 g, 41.85 mmol) in 40 ml of H₂O were added to the mixture. Excess acetic acid (16 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was basified with NaHCO₃ saturated solution and extracted with dichloromethane. The organic phase was dried over anhydrous magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (85/15 heptane/EtOAc) to yield 0.33 g (20%) of UB-88 as red oil (55/45 rotamers mixture). R.f. = 0.42 (Heptane/EtOAc 80/20); ¹H-NMR (CDCl₃, 400 MHz): 1.37 (s, 9H, C(CH₃)₃), 1.40-1.45 (m, 9H, C(CH₃)₃), 3.69 (s, 1.1H, CH₂), 3.83 (s, 0.9H, CH₂), 4.53 (s, 0.9H, CH₂), 4.59 (s, 1.1H, CH₂), 7.40-7.59 (m, 2H, Ar-H), 8.36-8.53 (m, 2H, Ar-H), 10.15 (s, 1H, Ar-H); ¹³C-NMR (CDCl₃, 100 MHz): 28.0, 28.2, 28.3, 49.0, 49.3, 51.1, 51.6, 80.6, 80.7, 81.6, 81.7, 127.29, 127.36, 127.39, 127.5, 129.6, 129.7, 131.8, 131.9, 132.2, 132.9, 155.6, 155.8, 157.8, 157.9, 165.3, 168.81, 168.85.

Example 124m**N-(3-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxymethanaminium 2,2,2-trifluoroacetate (UB-92)**

To a solution of tert-butyl 2-((3-(1,2,4,5-tetrazin-3-yl)benzyl)(tert-butoxycarbonyl)amino)acetate (0.14 g, 0.36 mmol) in DCM (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.08 g (62%) of UMB-92 as a pink solid. ¹H-NMR (MeOD, 400 MHz): 4.03 (s, 2H, CH₂), 4.46 (s, 2H, CH₂), 7.79 (t, J = 7.8 Hz, 1H, Ar-H), 7.85 (d, J = 7.8 Hz, 1H, Ar-H), 8.72 (d, J = 7.8 Hz, 1H, Ar-H), 8.79 (s, 1H, Ar-H), 10.41 (s, 1H, Ar-H); ¹³C-NMR (MeOD, 100 MHz): 46.4, 50.3, 128.9, 129.3, 130.0, 132.2, 133.3, 134.0, 158.1, 165.8, 167.3.

Example 125 Blocking assay

Establishing tumor xenografts in mice (same procedure for the pretargeted PET imaging)

All animal studies were approved by the Danish Animal Welfare Council, ministry of Justice. Five weeks old female nude BALB/c mice (Charles River, Sulzfeld, Germany) were allowed to acclimatize for one week with access to water and chow ad libitum. Human colon cancer cell line (LS174T; obtained from ATCC) was cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-Glutamine, 1% Sodium pyruvate, 1% non-essential amino acids, and 1% penicillin-streptomycin at 37 °C and 5% CO₂. Cells were harvested by trypsination at a confluence of 70–90% and subcutaneous tumors were established in the left flank of the 6 weeks old animals by inoculation of $\sim 5 \times 10^6$ LS174T cells resuspended in sterile PBS (100 μ L) and allowed to grow for 7–10 days. Tumors were measured using a caliper and the volume calculated using the formula volume = $\frac{1}{2}$ (length x width²).

Example 126 Blocking experiments

These blocking experiments were made using the method described in Example 125. Tumor-bearing animals were matched in groups based on their tumor volume (tumor volumes of ~ 100 – 300 mm³, $n = 3$ in each group) and were administered 100 μ g/100 μ L of CC49-TCO (100 μ g/100 μ L, ~ 7 TCO/mAb) per mouse. After 3 days, animals were injected with non-radioactive Tz (10–20 nmol). 2 hours later, they were administered with [¹¹¹In]Q (~ 13 MBq/100 μ L, 1–2 nmol) via the tail vein. Tz [¹¹¹In]Q was radiolabeled as described in literature⁹. The mice were euthanized after 22 h and tumor, blood, heart, lung, liver, spleen, kidney, and muscle were resected. All tissues were weighted and the radioactivity measured in a gamma counter (Wizard2, Perkin Elmer) and data was corrected for decay, tissue weight and injected amount of radioactivity. Figure 3 displays the results. Compounds RGV_52, RGV_55, UB-12, UB-19, UB-28, UB-37, UB-44, UB-39, UB-40, UB-50, UB-138, UB-80, UB-92, UB-108, UB-137 and UB-163 were tested. Results indicate that provide a H-tetrazine compound (with a rate constant of approximately 50.000-90.000 M⁻¹ s⁻¹ (determined with standard TCO at 37 °C in PBS) and a lipophilicity of clogD_{7.4} < -3 result in a normalized blocking effect > 70.

Example 127 Pretargeted Imaging with TCO-modified mAb (CC49-TCO)

Tumor-bearing animals were matched into 4 groups based on their tumor volume (tumor volumes of ~ 60 – 180 mm³, $n = 3$ in each group) and were administered with CC49-TCO (100 μ g/100 μ L, 6.7 nmol) per mouse. After 72 h, the animals were injected via the tail vein with ¹⁸F-labeled Tz (UB-108 and UB-137, respectively) (5–10 MBq/100 μ L) was injected via the tail

vein. The animals were PET/CT scanned 1 h p.i. of the ^{18}F -labeled Tz and then euthanized. Tumor and blood was collected, weighted and the radioactivity measured in a gamma counter. Data were corrected for decay, tissue weight and injected amount of radioactivity. Static PET images were acquired with an energy window of 350–650 KeV and a time resolution of 6 ns.

5 CT scans were acquired using 360 projections, 65 kV, 500 μA and 400 ms. PET images were reconstructed using a 3-dimensional maximum a posteriori algorithm with CT-based attenuation correction. PET and CT images were co-registered and analyzed using Inveon software (Siemens). The mean percentage of injected dose per grams of tissue (%ID/g) in the tissue volume was extracted by manually drawing regions of interest (ROI) on the entire tissue.

10 The PET image from a mouse being injected with UB108 is shown in Figure 12, whereas the PET image from a mouse being injected with UB137 is shown in Figure 13.

Radiochemistry Examples

Example 128 Production of [^{18}F]fluoride. [^{18}F]fluoride was produced via a (p,n)-reaction on a CTI Siemens cyclotron (Rigshospitalet, Denmark) by irradiating [^{18}O]H₂O with 11 MeV protons. An anion exchange resin (Sep-Pak Light Waters Accell Plus QMA cartridge) was washed with EtOH (10 mL), 90 mg/mL KOTf (aq) (10 mL) and water (10 mL) and dried with air. Then the aqueous [^{18}F]fluoride solution was passed through this exchange resin and the resin eluted with a mixture of KOTf (10 mg) and 50 μg K₂CO₃ in 550 μL water. The resulting

15 mixture was then gently concentrated to dryness at 100 °C by azeotropic drying with 2 x ACN (0.6 mL), under a nitrogen stream for 20 min, to give no-carrier-added K[^{18}F]F complex as a white semi-solid residue. Samples were analyzed by analytical HPLC as previously described, and radioactivity was analyzed using a flow-through GM tube based radiodetector (Scansys). Radiochemical conversion (RCC) of all radiolabeled compounds were determined by analytical

20 HPLC by comparison of the ^{19}F reference compounds synthesised previously. The radiochemical yield (RCY) was determined using the activity of [^{18}F]fluoride received from the cyclotron at the beginning of the synthesis and that of the purified product and the decomposition was corrected and have been decay corrected.

30 **Example 129 General Tetrazine radiolabeling (manual synthesis).** The preparation of the final compound, was performed using a method described previously, with minor modifications according to the optimization results described below.^[10] The organotin precursor (0.01 mmol) was dissolved in 0.8 mL DMA and added 0.1 mL of stock solutions of Cu(OTf)₂ (7.2 mg, 0.02 mmol in 0.1 mL DMA) and pyridine (12 μL , 0.15 mmol in 0.1 mL DMA). This mixture was

35 added to the dried [^{18}F]FK and heated to 100 °C for 5 min. The mixture was cooled down

before quenched with 1 mL of water. Samples were analysed via Radio-HPLC to determine the radiochemical conversion of [^{18}F]-Tz (decay corrected).

Example 130 General Tetrazine radiolabeling (automated synthesis). Automated synthesis was performed on a Scansys Laboratorieteknik synthesis module. The same procedure was used as in the optimization with minor differences. A solution of the organotin precursors (0.01 mmol), $\text{Cu}(\text{OTf})_2$ (7.2 mg, 0.02 mmol), and pyridine (12 μL , 0.15 mmol) in 1 mL DMA was added to a reaction vial containing the dried fluoride and the reaction allowed to proceed at 100 $^{\circ}\text{C}$ for 5 minutes. The solution was then cooled to 40 $^{\circ}\text{C}$ with compressed air before quenched with 2 mL of water. The crude reaction was then purified via semi-preparative HPLC (Thermo Fisher UltiMate 3000) with a C-18 column (Luna 5 μm C18(2) 100 $\text{\AA}$, 250 mm $\times$ 10 mm) used an isocratic method (with different H_2O :ACN solvent mixtures for each product, flowrate 4 mL/min). Identification and purity were determined by analytical HPLC.

Example 131 Labeling of UB108 and UB137

Production of [^{18}F]fluoride. [^{18}F]fluoride was produced via a (p,n)-reaction on a CTI Siemens cyclotron (Rigshospitalet, Denmark) by irradiating [^{18}O] H_2O with 11 MeV protons. An anion exchange resin (Sep-Pak Light Waters Accell Plus QMA cartridge) was washed with EtOH (10 mL), 90 mg/mL KOTf (aq) (10 mL) and water (10 mL) and dried with air. Then the aqueous [^{18}F]fluoride solution was passed through this exchange resin and the resin eluted with a mixture of KOTf (10 mg) and 50 μg K_2CO_3 in 550 μL water. The resulting mixture was then gently concentrated to dryness at 100 $^{\circ}\text{C}$ by azeotropic drying with 2 x ACN (0.6 mL), under a nitrogen stream for 20 min, to give no-carrier-added $\text{K}[^{18}\text{F}]\text{F}$ complex as a white semi-solid residue. Samples were analyzed by analytical HPLC as previously described, and radioactivity was analyzed using a flow-through GM tube based radiodetector (Scansys).

Example 132 Labeling of UB108 and UB137 (manual synthesis). The preparation of the final compound was performed using a method described previously, with minor modifications according to the optimization results described below.^[10] The organotin precursor (0.01 mmol) was dissolved in 0.8 mL DMA and added 0.1 mL of stock solutions of $\text{Cu}(\text{OTf})_2$ (7.2 mg, 0.02 mmol in 0.1 mL DMA) and pyridine (12 μL , 0.15 mmol in 0.1 mL DMA). This mixture was added to the dried [^{18}F]FK and heated to 100 $^{\circ}\text{C}$ for 5 min. The mixture was cooled down before quenched with 1 mL of water. The reaction mixture was diluted with 10 mL of H_2O and then put through a Sep-Pak Plus 18 cartridge (SPE) preconditioned by flushing with 10 mL of

EtOH followed by 10 mL of H₂O. The SPE was eluted with 3 mL of ACN into a vial containing 1 mL TFA. The mixture containing the protected product was heated during 15 minutes at 80 °C for fully deprotection. The mixture was cooled down and the sample was analysed via Radio-HPLC to determine the final radiochemical conversion of [¹⁸F]-Tz.

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Example 133 Labeling of UB-108 and UB-137 (automated synthesis for animal experiments). Automated synthesis was performed on a Scansys Laboratorieteknik synthesis module. The same procedure was used as in manual synthesis (Example 132) with minor differences. A solution of the organotin precursors (0.01 mmol), Cu(OTf)₂ (7.2 mg, 0.02 mmol), and pyridine (12 µL, 0.15 mmol) in 1 mL DMA was added to a reaction vial containing the dried fluoride and the reaction allowed to proceed at 100 °C for 5 minutes. The solution was then cooled to 40 °C with compressed air before quenched with 3 mL of water. The reaction mixture was diluted then put through a Sep-Pak Plus 18 cartridge (SPE) preconditioned by flushing with 10 mL of EtOH followed by 10 mL of H₂O. The SPE was wash with 10 mL water and dry with air, then was eluted with 3 mL of ACN into a vial containing 1 mL TFA. The mixture containing the protected product was heated during 15 minutes at 80 °C for fully deprotection. The crude was heated to 100 °C to evaporate the ACN/TFA mixture. The solution was then cooled to 40 °C with compressed air before the addition of 3 mL of water. The crude reaction was then purified via semi-preparative HPLC (Thermo Fisher UltiMate 3000) with a C-18 column (Luna 5 µm C18(2) 100 Å, 250 mm × 10 mm) using an isocratic method (15% EtOH in water 0.1% TFA, flowrate 4 mL/min). The collected fraction from HPLC was diluted with 0.1 M phosphate buffer to pH 7.4, and the concentration of radioactivity and ethanol was adjusted by diluting with saline solution to a final concentration of 100-50 MBq/mL and ≥ 5%vol. EtOH respectively. The automated synthesis including [¹⁸F]fluoride collection, azeotropic drying, labeling, HPLC separation and formulation was carried out within 90 minutes.

Example 134 TCO click ability.

To a solution of TCO 500 µL (1 mg/mL) PBS was added 100 µL of the formulated ¹⁸F-tetrazine (UB108/UB137). The solution was injected in the analytical HPLC to determine the fraction of tetrazines reacted to the TCO. All the tetrazines reacted immediately with the TCO (Figure 11).

Example 135 HPLC tests

Radio-HPLC tracer of the purified compounds, following the general procedure for the automated synthesis of the tetrazines describe in Example 130 and 133, with authentic UV references overlaid are shown below. The solid red line indicates the radio-HPLC trace and

the solid black line indicates the UV trace for the cold reference material. All samples were run using analytical HPLC method: Thermo Fisher UltiMate 3000 with a C-18 column (Luna 5 μ m C18(2) 100 Å, 150 mm $\times$ 4.6 mm). Eluents: A, H₂O with 0.1% TFA; B, MeCN with 0.1% TFA. Gradient from 100% A to 100% B over 12 minutes, back to 100% A over 3 min, flow rate 2 mL/min. Detection by UV absorption at λ = 254 nm on a UVD 170U detector.

Example 136 Calculation of cLogD_{7.4}

Distribution coefficient at physiological pH (7.4) were calculated in Chemicalize 2019, ChemAxon. Values are displayed in Figure 3.

10 Example 137 Synthesis of Q

Q was prepared as previously described in Rossin, R.; Verkerk, P.R.; van den Bosch, S.M.; Volders, R.C.; Verel, I.; Lub, J.; Robillard, M.S. In vivo chemistry for pretargeted tumor imaging in live mice. *Angew. Chem. Int. Ed.* 2010, 49, 3375–3378

Example 138 Rate Constant determinations

- 15 Reaction kinetics of the Tz-derivatives were determined by pseudo-first order measurements in dioxane at $25.0 \pm 0.1^\circ\text{C}$ or PBS at $27.0 \pm 0.1^\circ\text{C}$ in a SX20 stopped flow photometer (Applied Photophysics). The pseudo first order rate constant was determined by linearization of the decay curve followed by linear fitting. The second order rate constant was calculated from the pseudo-first order rate constant. Figure 3 displays the results.

20 Example 139 Pretargeted PET imaging

- The shelf stability of UB-108 was assessed in phosphate-buffered saline (PBS) by analytical-HPLC. UB-108 did not show degradation in PBS after 12 h at 37 °C at a concentration of 2 nmol/mL (98%). Consequently, the stannane precursor was synthesized in 4 steps (example 98-101). Radiolabeling succeeded in a one-pot, two-step sequence with a RCY (d.c.) of $11 \pm 3\%$ (n = 4) and an overall synthesis time of ca. 90 minutes including synthesis, separation and formulation. [¹⁸F]-UB108 was obtained with an Am of 134 ± 22 GBq/ μ mol (d.c.), a RCP of $\geq 99\%$ (n=4) and an activity yield of 600–700 MBq (EOS) starting from ~ 12 GBq fluoride-18. [¹⁸F]-UB108 was stable in PBS at room temperature for minimum 4 h and rapidly reacted with TCO-PNP carbonate (23) as confirmed by radio-HPLC. Residual amounts of Cu and Sn in the final formulated solution were analyzed by ICP-MS and found to be well below the

allowed limits specified in the ICH Guidelines (41–60 and 2.3–3.0 µg/L vs. 300 and 600 µg/day, respectively).

The performance of [¹⁸F]-UB108 as prepared in Example 97 was tested in pretargeted PET imaging (Figure 14A). Balb/c nude mice bearing LS174T tumor xenografts (n = 3 per group) were injected i.v. with either CC49-TCO (100 µg, 3.9 nmol, ~7 TCO/mAb) or non-modified CC49 (control). After 72 h, [¹⁸F]-UB108 (2.86 ± 0.99 MBq /100 µL) was administered and the mice were PET/CT scanned after 1 h. Image-derived uptake in tumor, heart (surrogate for blood) and muscle tissue was quantified as percentage injected dose per gram (mean % ID/g), (Figure 14B, C, D and E). After completion of the scan, mice were euthanized and ex vivo biodistribution was performed. After completion of the scan, the mice were euthanized and different tissues were resected, weighted, and the radioactivity measured using a gamma counter (Wizard2, Perkin Elmer). Data was corrected for decay, tissue weight and injected amount of radioactivity. Mice pretreated with CC49-TCO demonstrated a mean tumor uptake of [¹⁸F]-UB108 of 0.99 ± 0.14 %ID/g (mean ± S.E.M.). The tracer displayed good target-to-background ratios with muscle uptake < 0.15 %ID/g for all animals (Table 3). This was also evident from PET/CT images, where tumor uptake in the CC49-TCO group was clearly visible (Figure 14E). The mean tumor-to-blood ratio was 0.9, and thereby the specific uptake is similar to what was previously reported for other pretargeted imaging agents in the same tumor model.^[21] In contrast, a mean tumor-to-muscle ratio of 10 was detected which in fact is significantly higher compared to what has previously been found for the “state-of-the-art” Tz-based imaging agents [¹¹¹In]Q and [⁶⁴Cu]Cu-NOTA-PEG7-H-Tz - in a similar pretargeting set-up (LS174T bearing mice, using CC49-TCO 72h prior to tracer injection, similar imaging timeframes) (Figure 14C and D). However, [¹⁸F]-UB108 showed a 3 to 5-fold lower tumor uptake compared to those imaging agents (Figure 14E). All tissues including tumors showed low ¹⁸F-uptake in control animals (CC49) (tumor uptake of 0.05 ± 0.04 %ID/g). The findings from the imaging experiment were confirmed by ex vivo biodistribution data (Table 4). Except for the tumor, the only tissue, where the tracer uptake was significant, was blood. This accumulation is likely caused by the *in vivo* ligation of [¹⁸F]-UB108 to CC49-TCO still circulating in the bloodstream. An observation that has been reported before for other pretargeting pairs. If residual mAbs are removed from the blood pool by e.g. a clearing agent, subsequent injection of [¹⁸F]-UB108 will likely result in an improved tumor-to-blood ratio.

Example 140 testing in vivo applicability of tetrazines with different lipophilicity

A structurally diverse library of 45 Tz-derivatives was prepared (Figure 15), covering a wide spectrum of physicochemical properties, in particular calculated TPSAs between 60–350 Å² and different lipophilicities, with calculated logD_{7.4} values (clogD_{7.4}) ranging from approximately -7.0 to 2.5 (Table 5). The Tz-scaffolds (A–L) include mono- and disubstituted methyl-, phenyl-, 2-pyrimidyl-, and 2-pyridyl-substituted Tz-derivatives with second-order rate constants for the reaction with TCO ranging from 1.4 to 230 M⁻¹ s⁻¹ in 1,4-dioxane at 25 °C, and from 1,100 to 73,000 M⁻¹ s⁻¹ in buffered aqueous solution at 37 °C. Figure 15 provides an overview of the synthesized Tz-library and Table 5 displays the measured rate constants and calculated physicochemical properties of each Tz. Several compounds were obtained as copper(II) complexes (for details see the Supporting Information), which was taken into account in the calculation of clogD_{7.4} and TPSA. The distribution coefficient at physiological pH (logD_{7.4}) and TPSA were calculated using the software Chemicalize. Tetrazines conjugated to DOTA were calculated with chelated trivalent cations, Tzs with other chelators with bivalent cations. Second-order rate constants for the Tz scaffolds A-L were determined by stopped-flow spectrophotometry (n≥4), monitoring the reaction of representative tetrazines with unsubstituted trans-cyclooctene (TCO) at 25 °C in 1,4-dioxane, and with TCO-PEG4 (modified TCO-5ax-OH, 'minor-TCO') in Dulbecco's phosphate buffered saline (DBPS) at 37 °C. n≥3.

The assay was inspired by receptor blocking experiments and based on the pretargeted imaging approach reported by Rossin et al. An ¹¹¹In-labeled Tz [¹¹¹In]Q was used in pair with TCO-modified CC49, a non-internalizing mAb that targets the tumor-associated glycoprotein 72 (TAG72), as a benchmark model for the in vivo ligation.

The TCO-modification of CC49 was carried out according to Rossin et al. To study the in vivo ligation performance of Tz-derivatives 1a–45a, BALB/c mice bearing LS174T colon carcinoma xenografts were injected intravenously (i.v.) with CC49-TCO 72 h prior to i.v. injection of the unlabeled Tz, followed by administration of [¹¹¹In]Q 1 h later. The animals were euthanized after 22 h and an ex vivo biodistribution was carried out to quantify the tumor uptake of [¹¹¹In]Q (Figure 15). The efficiency of the in vivo ligation of the unlabeled Tz can thus be correlated to a reduced uptake of [¹¹¹In]Q (Fig. 15). As a control, blocking was performed using the non-radioactive precursor of [¹¹¹In]Q, which blocked ≥99% of the [¹¹¹In]Q tumor uptake. A group of CC49-TCO pretreated mice were injected exclusively with [¹¹¹In]Q (without blocking) and the determined uptake was used as reference value (100%) to normalize the observed changes in tumor uptake in blocking experiments.

Figure 16A displays the blocking assay and Figure 16B summarizes the results for the entire Tz-library in the assay. The highest blocking efficiencies (95–99%) were observed for the Tz-chelator conjugates 4a, 24a, 35a and **Q**, the Tz-carboxylic acids 33a and 39a, the Tz-PEG derivative 40a, and the Tz-sugar conjugate 45a. All of these probes include H-phenyl-, pyrimidyl-phenyl-, or bis(pyridyl)-Tz-scaffolds with second-order rate constants for the reaction with TCO of $>70 \text{ M}^{-1} \text{ s}^{-1}$ (1,4-dioxane, 25 °C) or of $>39,000 \text{ M}^{-1} \text{ s}^{-1}$ (DPBS, 37 °C) (cf. Table 5). Next, potential correlations between the blocking effect, the $\text{clogD}_{7.4}$ and the TPSA, as well as IEDDA reactivity were investigated. High IEDDA reactivity was shown to directly correlate with the blocking effect and thus confirmed to be a key parameter for the in vivo ligation performance of Tz derivatives (Fig. 16C, 16D). We did not observe a strong correlation between the blocking effect and TPSA. However, a distinct relationship between $\text{clogD}_{7.4}$ values and the blocking effect was evidently observed when comparing Tz-derivatives with similar IEDDA reactivity (Fig. 16C, 16D). For all Tz-scaffolds a Pearson's correlation coefficient >0.78 was found. Our results show that high IEDDA reactivity ($>50,000 \text{ M}^{-1} \text{ s}^{-1}$) and a $\text{clogD}_{7.4}$ of -3 or lower are strong indicators for high in vivo ligation performance of Tz-derivatives in pretargeting approaches using the described tumor model (Figure 16A). The blocking effect of non-radiolabeled Tz was determined as the change in tumor uptake of [^{111}In]**Q** 22 h p.i. Each Tz was administered 1 h prior to [^{111}In]**Q** and the uptake normalized to a group of animals where no blocking was performed (control). Data represent mean from $n = 3$ mice/group. Correlation of blocking effect and $\text{clogD}_{7.4}$ for Tz-derivatives with similar IEDDA reactivity (as shown in Figure 16C and D) where data was fitted to exponential growth equation $Y = Y_0 e^{kx}$ (dotted line). The statistical analysis of the correlation between tumor uptake and $\text{clogD}_{7.4}$ for the different groups of Tz-derivatives (shown in Table 7) was obtained by Pearson's correlation coefficient (r) which describes the goodness of fit between the blocking effect and $\text{clogD}_{7.4}$. Notes: ^a Reaction of representative Tz with unsubstituted TCO; ^b Reaction of representative Tz with TCO-PEG4; ^c Measured for Tz-scaffold A only; n.d. = not determined.

Experimental design of a [^{18}F]Tz library

In order to verify that the results from the blocking study can be used to predict the outcome for in vivo PET imaging, 18 Tz-derivatives from the first library were selected. The selection was based on criteria such as, the possibility for ^{18}F -labeling, structural diversity, lipophilicities and distinct IEDDA reactivities. For this purpose, we decided to use an indirect radiolabeling approach, enabling the combination of different building blocks to rapidly access a series of ^{18}F -labeled Tz-derivatives. The Cu-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) appeared to be suitable in this respect, as it allows for fast and

efficient radiolabeling under mild reaction conditions. Six precursor Tz-alkynes (I–VI) were prepared and reacted with three ^{18}F -labeled azides ($[^{18}\text{F}]\text{Az1–Az3}$) to obtain 18 different $[^{18}\text{F}]\text{Tz}$ -probes (Figure 17). Notes to Table 6 showing the results obtained: ^aRCYs were decay-corrected to the starting amount of radioactivity for the respective azide or ^{*}RCY was determined starting from ^{18}F -. ^bMolar activities (A_m) differ due to the use of different cyclotrons. ^cRCP was determined by radio-HPLC. ^dIn vivo stability of $[^{18}\text{F}]\text{Tz}$ was assessed by determining the fraction (%) of radioactivity corresponding to intact compound after 30 min ($n = 4$) from radio-TLC analysis. n.d. = not determined.

Indirect ^{18}F -labeling of a Tz series via Cu-catalyzed click chemistry

Azide building blocks were ^{18}F -labeled using fully automated procedures to afford $[^{18}\text{F}]\text{Az1–Az3}$ and Tz-alkynes I–VI were synthesized. Subsequent radiolabeling via the CuAAC was achieved in various yields, up to approximately 70% (Table 6). Applied conditions for the CuAAC differed depending on the substituents attached to the Tz-scaffold. In general, radiolabeling was carried out at room temperature with reaction times of 10–15 min using aqueous solutions of CuSO_4 , sodium ascorbate and disodium bathophenanthroline disulfonate (BPDS). For the synthesis of the bis(pyridyl)Tz-derivatives $[^{18}\text{F}]\text{25a}$, $[^{18}\text{F}]\text{44a}$ and $[^{18}\text{F}]\text{45a}$, increased amounts of the catalysts, longer reaction times (20–25 min) and elevated temperatures (120 °C) were required in order to reach radiochemical conversions (RCCs) of $\geq 70\%$. A possible reason for the harsher conditions required for this scaffold may be a result from coordination of Cu by the bis(pyridyl)-Tz moiety. Radiochemical yields (RCYs) and molar activities (A_m) for all ^{18}F -labeled Tz-derivatives are presented in Table 6. Radiochemical purities (RCPs) of the isolated compounds were high ($>90\%$), except for $[^{18}\text{F}]\text{25a}$ and $[^{18}\text{F}]\text{26a}$ (83–85%) due to radiolysis (observed for $[^{18}\text{F}]\text{25a}$), undesired decomposition, and difficult separation of the resulting byproducts. During the radiolabeling partial reduction of $[^{18}\text{F}]\text{19a}$ and $[^{18}\text{F}]\text{44a}$ to the corresponding dihydro-Tz was observed. However, these Tz-derivatives were reoxidized using phenyliodonium diacetate (PIDA). In the case of $[^{18}\text{F}]\text{44a}$, complete reduction to the dihydro-Tz (using ascorbic acid) and reoxidation upon purification was applied to prevent radiolysis. Excess PIDA and byproducts were removed during solid-phase extraction to obtain $[^{18}\text{F}]\text{44a}$ in a RCP of 98% (Table 6). Moreover, during the synthesis of $[^{18}\text{F}]\text{45a}$ an alternative deprotection method for the azide ($[^{18}\text{F}]\text{Az3}$) was required to avoid decomposition of the Tz-scaffold during the CuAAC. All ^{18}F -labeled Tz-derivatives were formulated in 0.9% saline prior further studies. Overall, radiofluorination via the CuAAC allowed for the preparation of a structurally diverse series of ^{18}F -labeled Tz-derivatives. In contrast to routinely used direct radiofluorination methods, this

building block approach gave access to highly reactive [^{18}F]Tz-probes, using Tz-scaffolds that have previously been reported to be inaccessible.

In vivo stability of ^{18}F -labeled Tz-derivatives in naïve mice

Next, we investigated whether there is a relationship between the *in vivo* stability of Tz-derivatives and their blocking ability. Fifteen ^{18}F -labeled tetrazines were studied in naïve mice. Blood was collected after 30 min, and plasma samples were analyzed by radio-TLC for stability assessment (Table 6). Interestingly, the *in vivo* stability had only a limited or even no effect on the *in vivo* ligation performance as evaluated in the blocking study (cf. Table 5). Consequently, six [^{18}F]Tz ([^{18}F]1a, [^{18}F]3a, [^{18}F]19a, [^{18}F]26a, [^{18}F]44a, and [^{18}F]45a) were selected for further *in vivo* studies solely based on the IEDDA-reactivity (second-order rate constants between 72 and 230 $\text{M}^{-1}\text{s}^{-1}$) and lipophilicity ($\text{clogD}_{7.4}$ between -1.53 and 2.10). These radiolabeled Tz-probes were used to investigate if the results from the blocking assay can be translated to pretargeted PET imaging at tracer doses.

Pretargeted PET imaging

Of the six Tz-probes selected for evaluation in pretargeted PET imaging studies, four compounds (3a, 19a, 44a and 45a) showed a good to excellent blocking effect (72–99%), while two probes (1a and 26a) only showed limited effect (9% for 1a and 30% for 26a). The latter were included to verify that blocking results can reliably be used to predict the capability of radiolabeled Tz for pretargeted *in vivo* chemistry.

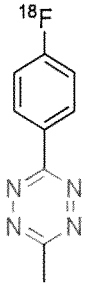
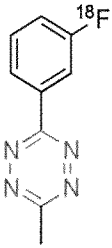
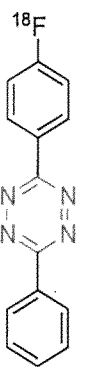
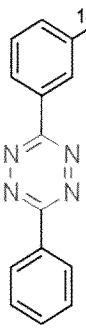
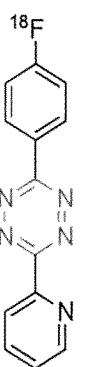
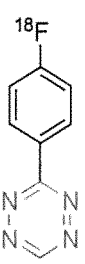
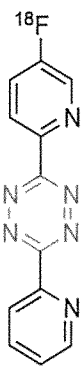
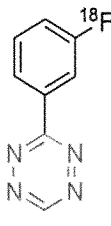
Mice ($n = 3\text{--}4$ per group) were injected i.v. with either CC49-TCO or 0.9% saline (control experiments). After 72 h, ^{18}F -labeled Tz (5–10 MBq) were administered and the mice were PET/CT-scanned 1 h p.i. A 3D region of interest (ROI) was created on the entire tumor volume, as well as heart and muscle tissue, and the uptake was quantified as percentage of the injected dose per gram (mean % ID/g), tumor-to-blood (T/B) and tumor-to-muscle (T/M) ratios (Fig. 18A-D). Figure 18 (A) is a schematic illustration of the pretargeting experiment and the research question: Is there a correlation between the blocking effect and the PET imaging contrast. (B) Image-derived mean uptake values are presented as percentage of the injected dose per gram (%ID/g) in tumor. (C) Tumor-to-muscle (T/M) ratios ($n = 4$ mice, except for [^{18}F]3, $n = 3$). Data is represented as mean $\pm$ S.D. (* $p < 0.05$ and *** $p < 0.001$). For all groups $n = 4$ mice (except [^{18}F]3a where $n = 3$) (D) Correlation between the blocking effect of the unlabeled Tz-derivatives 1a, 3a, 19a, 26a, 44a, and 45a and the T/M-ratios for the corresponding ^{18}F -labeled compounds observed by *in vivo* pretargeted PET imaging. A strong correlation was found (linear regression: $R^2 = 0.78$, $p = 0.019$, $n = 6$). Data is represented as mean $\pm$ S. D. The asterisk indicates significant difference (* $p < 0.05$ and ***

$p < 0.001$) when compared to control. (E) Representative images of PET scans 1 h p.i. of the radiolabeled Tz ($[^{18}\text{F}]1\text{a}$, $[^{18}\text{F}]3\text{a}$, $[^{18}\text{F}]19\text{a}$, $[^{18}\text{F}]26\text{a}$, $[^{18}\text{F}]44\text{a}$ and $[^{18}\text{F}]45\text{a}$) in pretargeted PET imaging studies (T indicates the position of the tumor). $[^{18}\text{F}]3\text{a}$, $[^{18}\text{F}]19\text{a}$ and $[^{18}\text{F}]45\text{a}$ displayed specific tumor uptake and the tumor is clearly visualized in the PET image.

- 5 The tumor uptake of the different ^{18}F -labeled Tz-probes was at a rather similar level, however, $[^{18}\text{F}]3\text{a}$, $[^{18}\text{F}]19\text{a}$ and $[^{18}\text{F}]45\text{a}$ showed a significantly increased tumor accumulation in mice pretreated with CC49-TCO compared to control animals (Figure 18A and 18E). In accordance with blocking results, no significant difference in tumor uptake between pretargeting experiments and controls for $[^{18}\text{F}]1\text{a}$ and $[^{18}\text{F}]26\text{a}$. In the case of $[^{18}\text{F}]44\text{a}$, no
10 increase in tumor accumulation was observed in mice pretreated with TCO-modified mAb. However, this Tz showed higher radioactivity levels in the heart in mice pretreated with CC49-TCO compared to controls (2.5 ± 0.7 %ID/g and 1.5 ± 0.2 %ID/g, respectively; Figure 18B). This indicates that $[^{18}\text{F}]44\text{a}$ binds to mAb still circulating in the blood pool. This difference was also observed for the three ^{18}F -labeled Tz-derivatives showing specific tumor
15 accumulation ($[^{18}\text{F}]3\text{a}$, $[^{18}\text{F}]19\text{a}$ and $[^{18}\text{F}]45\text{a}$), but not for the non-accumulating probes $[^{18}\text{F}]1\text{a}$ and $[^{18}\text{F}]26\text{a}$ (Figure 18B). Binding to residual mAb in the blood pool is a frequently reported challenge in pretargeted imaging approaches, and has been addressed by the development of clearing agents. However, ligation in blood did not hinder the investigation of the *in vivo* ligation performance of ^{18}F -labeled Tz-probes in comparison to blocking efficiencies.
- 20 Finally, the relationship between the *in vivo* performance of the used Tz-probes and the results obtained from the blocking assay was investigated. A strong correlation was found between the blocking effect of the unlabeled Tz and the T/M ratio (Figure 18D) as well as the selective tumor uptake (tumor to tumor-control (T/Tc) ratio) of the respective ^{18}F -labeled probes in pretargeted PET imaging studies. These significant relationships confirm the
25 validity of the blocking assay, and our finding that reduced lipophilicity and high IEDDA reactivity are key parameters for the *in vivo* performance of Tz-derivatives. Our results show that low lipophilicity enhances the ability of the bioorthogonal Tz-agent to bind to TCO-mAbs at the tumor site. Moreover, faster excretion of radiolabeled probes, which is crucial for obtaining high tumor-to-background ratios is also facilitated by low lipophilicity of Tz-
30 derivatives (Figure 18).

Tables

Table 1. Product scope regarding the tetrazine scaffold, relative to the optimized radiolabeling condition of the Cu-mediated ^{18}F -fluorination reaction.

									Reactivity increase
	$[^{18}\text{F}]\text{Tz1}$	$[^{18}\text{F}]\text{W}$	$[^{18}\text{F}]\text{E}$	$[^{18}\text{F}]\text{R}$	$[^{18}\text{F}]\text{T}$	$[^{18}\text{F}]\text{Y}$	$[^{18}\text{F}]\text{U}$	$[^{18}\text{F}]\text{-9}$	
RCC ^[a] (%)	30±5	28±1	30±5	31±2	_[d]	18±4	_[d]	11±1	
RCY ^[b] (%)	23±1	26±2	23±2	24±3	_[d]		_[d]	11±3	
k_2 [$\text{M}^{-1}\text{s}^{-1}$] ^[c]	2.25	nd	18	30	106	159	206	222	
RCP ^[a] (%)	≥99	≥99	≥99	≥99	_[d]	99	_[d]	99	

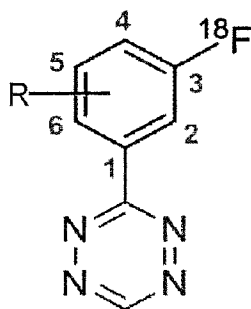
[a] Radiochemical conversion (RCC) and Radiochemical purity (RCP) were determined by radio-HPLC (n=3).

[b] Radiochemical yield (RCY) was decay corrected to the starting amount of radioactivity received from the cyclotron and the isolated product without formulation step (n=3).

[c] Second order rate constants estimated from stopped-flow measurements of the alkyne-Tz building block with TCO at 25°C in ACN.

[d] No product formed or could not be isolated.

Table 2. Product scope in respect to the substitution pattern when including different linkers in the aromatic ring, relative to the Cu-mediated ^{18}F -fluorination reaction.



Substituents	Position			Position		
	4	5	6	4	5	6
-CH ₃	_[a]	14±3 ^[d]	_[c]	UB-007	UB-052	RGV_117
-OCH ₃	4±1	17±3 ^[d]	_[c]	UB-008	UB-048	RGV_116
-NHCOCH ₃	_[a]	31±3 ^[d]	_[b]	UB-148	UB-150	-
-CONHCH ₃	_[a]	_[d]	_[b]	UB-198	UB-200	-
-CONH ₂	_[a]	_[d]	_[b]	UB-22	UB-70	-

[a] Stannate precursor could not be synthesized.

[b] No tetrazine formation detected.

[c] Decomposition during Cu-mediated ^{18}F -fluorination reaction.

[d] RCCs were determined by radio-HPLC (n = 3).

Table 3 Image derived uptake values of [^{18}F]UB-108 in pretargeted experiments

	CC49-TCO	CC49
	(mean %ID/g) n = 3	(mean %ID/g) n = 3
Tumor	0.99 ± 0.15	0.05 ± 0.04
Heart	1.15 ± 0.16	0.04 ± 0.00
Muscle	0.09 ± 0.04	0.02 ± 0.02

Table 4. Wellcounter data of ex vivo uptake values of [18F]UB-108 in pretargeted experiments.

	CC49-TCO (mean %ID/g). <i>n</i> = 3	CC49 (mean %ID/g). <i>n</i> = 3
Tumor	1.85 ± 0.21	0.06 ± 0.02
Blood	1.25 ± 0.02	0.06 ± 0.00
Heart	0.27 ± 0.01	0.02 ± 0.00
Lung	0.51 ± 0.03	0.06 ± 0.00
Liver	0.49 ± 0.02	0.10 ± 0.01
Spleen	0.32 ± 0.02	0.02 ± 0.00
Kidney	0.47 ± 0.02	0.26 ± 0.01
Muscle	0.12 ± 0.03	0.03 ± 0.02

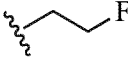
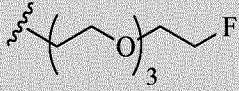
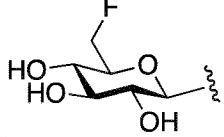
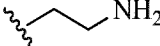
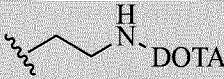
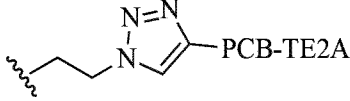
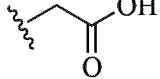
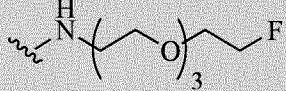
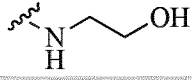
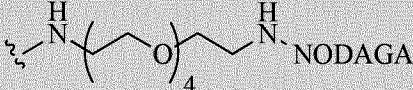
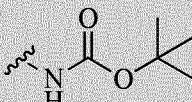
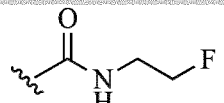
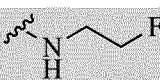
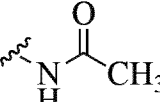
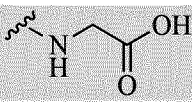
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Table 5. Calculated physicochemical properties (TPSA and clogD_{7.4}), measured second-order rate constants for the ligation with TCO, and blocking efficiencies of the Tz-derivatives shown in Figure 15.

Tz	Tz-scaffold	R	clogD _{7.4} ^a	TPSA ^a (Å ²)	Rate constant (1,4-dioxane, 25 °C, M ⁻¹ s ⁻¹) ^b	Rate constant (DPBS, 37 °C, M ⁻¹ s ⁻¹) ^b	Blocking effect (%) ^c	% Tumor Uptake of [¹¹¹ In]46 after blocking
1a	A	I	0.29	91	72	39,000	9	91
2a	A	II	-0.06	119	72	39,000	4	96
3a	A	III	-1.53	161	72	39,000	80	20
4a	A	IV	-2.86	217	72	39,000	95	5
5a	B	I	0.89	91	1.4	1,100	9	91
6a	B	II	0.54	119	1.4	1,100	1	99
7a	B	III	-0.93	161	1.4	1,100	13	87
8a	B	IV	-5.81	221	1.4	1,100	49	51
9a	C	V	-2.19	114	1.4	1,100	14	86
10a	C	VI	-2.05	214	1.4	1,100	32	68
11a	C	VII	-3.2	184	1.4	1,100	34	66
12a	D	I	1.89	104	13	<i>n.d.</i>	0	100
13a	D	II	1.53	132	13	<i>n.d.</i>	0	100
14a	D	III	0.06	174	13	<i>n.d.</i>	24	76
15a	D	VIII	-1.98	111	13	<i>n.d.</i>	78	22

17a	E	I	0.65	82	200	60,000	0	100
18a	E	II	0.29	109	200	60,000	28	72
19a	E	III	-1.18	152	200	60,000	76	24
20a	E	IV	-2.46	208	200	60,000	94	6
21a	F	X	-1.97	114	83	<i>n.d.</i>	87	13
22a	F	XI	0.64	134	83	<i>n.d.</i>	27	73
23a	F	XII	0.12	126	83	<i>n.d.</i>	72	28
24a	F	XIII	-4.2	278	83	<i>n.d.</i>	96	4
25a	G	I	2.46	137	230	73,000	16	84
26a	G	II	2.10	164	230	73,000	30	70
27a	G	III	0.63	207	230	73,000	51	49
28a	H	XIV	1.11	90	210	58,000	45	55
29a	H	XV	-0.14	78	210	58,000	55	45
30a	H	XVI	-0.18	63	210	58,000	62	38
31a	H	XVII	-0.41	80	210	58,000	60	40
32a	H	XVIII	-3.03	100	210	58,000	86	14
33a	H	XIX	-6.89	129	210	58,000	97	3
34a	H	XX	-4.4	202	210	58,000	95	5
35a ^d	H	XXI	-5.16	320	210	58,000	96	4
36a	I	-	1.79	61	10	<i>n.d.</i>	0	100
37a	K	XXII	-0.77	129	230	73,000	76	24
38a	K	XXIII	-2.23	161	230	73,000	83	17
39a	K	XXIV	-2.37	143	230	73,000	96	4
40a	K	XXV	-3.81	263	230	73,000	97	3
Q	K	XXVI	-4.13	362	230	73,000	99	1
42a	K	XXVII	1.62	129	230	73,000	75	25
43a	L	I	0.84	137	230	73,000	67	33
44a	L	II	0.48	164	230	73,000	72	28
45a	L	III	-0.99	207	230	73,000	99	1

Notes: ^aThe distribution coefficient at physiological pH ($\log D_{7.4}$) and TPSA were calculated using the software Chemicalize. Tetrazines conjugated to DOTA were calculated with chelated trivalent cations, Tzs with other chelators with bivalent cations. ^bSecond-order rate constants for the Tz scaffolds A-L were determined by stopped-flow spectrophotometry ($n \geq 4$), monitoring the reaction of representative tetrazines with unsubstituted *trans*-cyclooctene (TCO) at 25 °C in 1,4-dioxane, and with TCO-PEG₄ (modified TCO-5ax-OH, '*minor-TCO*') in Dulbecco's phosphate buffered saline (DBPS) at 37 °C. ^c $n \geq 3$; (see Supporting Information, Table S1 and Table S2). ^dData gained from Molecules 2021, 26, 544.

R	Structure
I	
II	
III	
IV	
V	
VI	
VII	
VIII	
IX	
X	
XI	
XII	
XIII	
XIV	
XV	
XVI	
XVII	
XVIII	

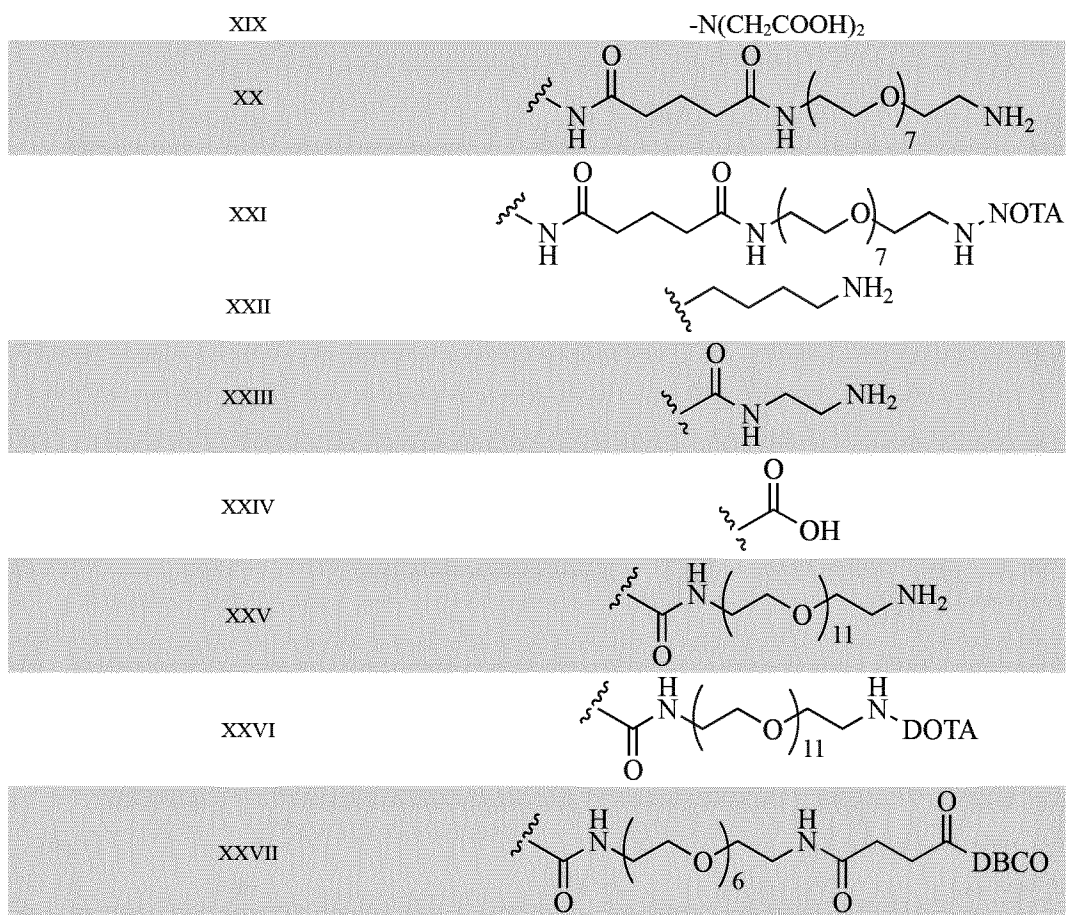


Table 6. Radiochemical yields (RCYs), molar activities (A_m) and radiochemical purities (RCPs) for the ^{18}F -labeled Tz-derivatives tested in Example 140 and as shown in Figure 17.

Tetrazine	Tz-alkyne (I-VI)	Azide-functionalized ^{18}F -click-agent	RCY (%) ^a	A_m^b (GBq/ μmol)	RCP (%) ^c	<i>In vivo</i> stability ^d (% intact [^{18}F]Tz after 30 min)	Blocking effect (%) (of unlabeled Tz)
[^{18}F]1a	IV	[^{18}F]Az1	25 ^c	55	99	90	9
[^{18}F]2a	IV	[^{18}F]Az2	23	22	96	37	4
[^{18}F]3a	IV	[^{18}F]Az3	61	31	98	76	81
[^{18}F]5a	VI	[^{18}F]Az1	14 [*]	106	≥ 99	26	10
[^{18}F]6a	VI	[^{18}F]Az2	33	100	≥ 99	85	1
[^{18}F]7a	VI	[^{18}F]Az3	52	230	≥ 99	60	3
[^{18}F]12a	V	[^{18}F]Az1	1 [*]	107	96	10	0
[^{18}F]13a	V	[^{18}F]Az2	11	21	94	16	0
[^{18}F]14a	V	[^{18}F]Az3	68	102	98	43	24
[^{18}F]17a	III	[^{18}F]Az1	8 [*]	209	98	32	0
[^{18}F]18a	III	[^{18}F]Az2	17	37	92	22	29
[^{18}F]19a	III	[^{18}F]Az3	59	29	98	87	76
[^{18}F]25a	II	[^{18}F]Az1	16 [*]	n.d.	83	n.d.	16
[^{18}F]26a	II	[^{18}F]Az2	36	54	≥ 85	27	30
[^{18}F]27a	II	[^{18}F]Az3	18 [*]	n.d.	≥ 90	n.d.	51
[^{18}F]43a	I	[^{18}F]Az1	1	5	90	10	67
[^{18}F]44a	I	[^{18}F]Az2	20	85	98	n.d.	72
[^{18}F]45a	I	[^{18}F]Az3	11	151	≥ 90	42	99

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Table 7: Statistical analysis of the correlation between tumor uptake and $\log D_{7.4}$ for the different groups of Tz-derivatives shown in Figure 16C and 16D.

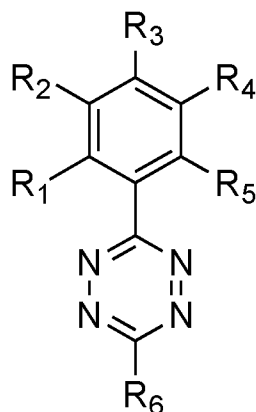
Tz-scaffolds grouped by IEDDA reactivity (second-order rate constants, $\text{M}^{-1}\text{s}^{-1}$)					
Tz-scaffolds (see Figure 16C, 16D)	B,C	D,I	A,F	E,H	G,L,K
1,4-dioxane, 25 °C ^a	1.4	10-13	72-83	200-210	230
DPBS buffer, 37 °C ^b	1,100	n.d.	39,000 ^c	58,000-60,000	73,000
Pearson's correlation coefficient (<i>r</i>)	0.92	0.89	0.78	0.94	0.86
<i>p</i> -value	0.003	0.017	0.021	< 0.0001	< 0.001
<i>n</i>	7	6	8	12	12

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- 25

Claims

1. A H-tetrazine compound, having the following formula I:



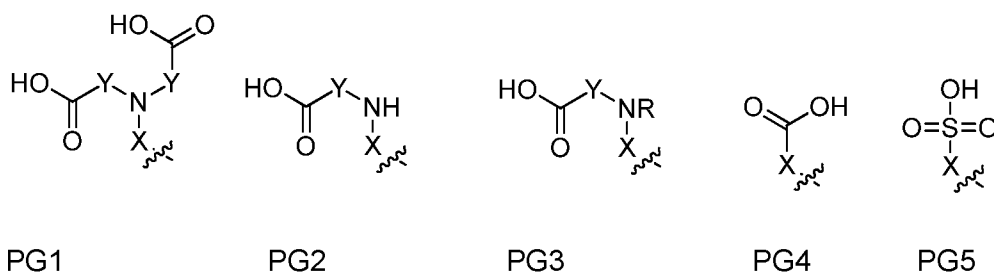
Formula I

wherein R₁-R₅ are independently selected from: a radionuclide selected from ¹⁸F, ¹²³I, ¹²⁴I, ¹³¹I or ²¹¹At; one or more group(s) providing a lipophilicity of clogD_{7.4} < -3 to the compound of Formula I independently selected from the group consisting of a hydroxy group, a sulfonamide, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine; and wherein R₆ is H;

wherein one of R₁-R₅ is a radionuclide and one or more of the remaining R₁-R₅ is a/are

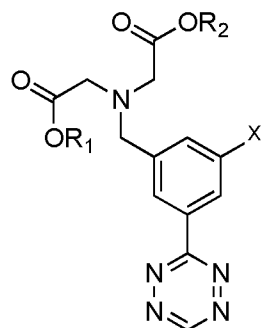
group(s) providing a lipophilicity of $\text{clogD}_{7.4} < -3$, and any R_1 - R_5 remaining thereafter is/are H.

2. A H-tetrazine compound according to claim 1, wherein said one or more group(s) providing a lipophilicity of $\text{clogD}_{7.4} < -3$ to the compound of Formula I is selected from: -OH, NR_7R_8 , $\text{CH}_2\text{N}(\text{CH}_2\text{COOH})_2$, $\text{CH}_2\text{NHCH}_2\text{COOH}$, $\text{CH}_2\text{NRCH}_2\text{COOH}$, CONR_7R_8 , SO_3H , SO_2NH_2 , and SO_2NH , wherein R is H, CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH , R_7 is H, CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH ; and R_8 is H, CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH .
3. A H-tetrazine compound according to claim 2, wherein said group providing lipophilicity of $\text{clogD}_{7.4} < -3$ is selected from $\text{CH}_2\text{N}(\text{CH}_2\text{COOH})_2$, $\text{CH}_2\text{NHCH}_2\text{COOH}$, $\text{CH}_2\text{NRCH}_2\text{COOH}$, wherein R is H, CH_3 , CH_2CH_3 , $\text{CH}_2\text{CH}_2\text{CH}_3$ or CH_2COOH , and is situated at position R_2 or R_4 in formula I; and wherein said radionuclide is situated at the other position R_2 or R_4 in Formula I.
4. A H-tetrazine compound according to any of the preceding claims, wherein said group providing a lipophilicity of $\text{clogD}_{7.4} < -3$ to the compound of Formula I is selected from the polar groups (PG):

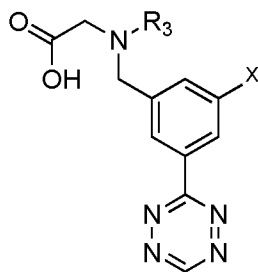


wherein X is $\text{CO}(\text{CH}_2)_n$, $\text{SO}_2(\text{CH}_2)_n$, $(\text{CH}_2)_n$ or $(\text{OCH}_2\text{CH}_2)_n$ and $n = 0, 1, 2$ or 3 ; and wherein Y is $(\text{CH}_2)_n$, $(\text{OCH}_2\text{CH}_2)_n$ or $\text{CO}(\text{CH}_2)_n$ and $n = 0, 1, 2$ or 3 ; and wherein R is $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOH}$, $(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{COOH}$ and $n = 0, 1, 2$ or 3 ; and wherein the curly sign indicates the link to the aromatic ring.

5. A H-tetrazine compound according to claim 1 selected from:



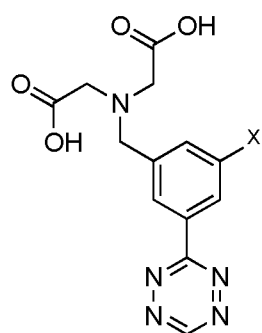
Ia



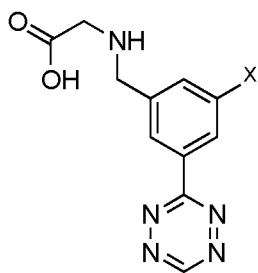
Ib

wherein X is selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At , and R_1 , R_2 and R_3 is independently
 5 selected from H, $(\text{CH}_2)_n\text{CH}_3$, $(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $\text{CO}(\text{CH}_2)_n\text{COOH}$, $(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{COOH}$
 and $n = 0, 1, 2$ or 3 .

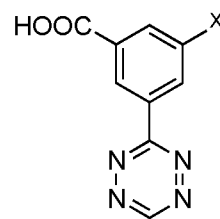
6. A H-tetrazine tetrazine according to claim 1 selected from:



Ic



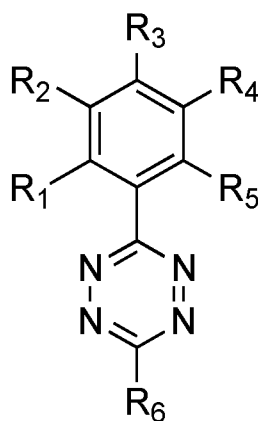
Id



Ie

wherein X is selected from ^{18}F , ^{123}I , ^{124}I , ^{131}I or ^{211}At .

7. A tetrazine precursor having the following formula II:



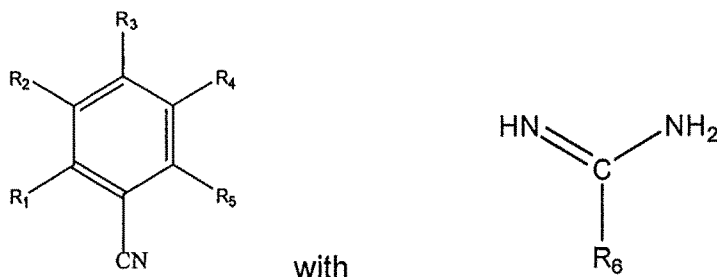
Formula II

wherein one of R₁-R₅ are independently selected from the group consisting of: SnR₃, B(OR)₂, I⁺-Ar, I double-bonded to R or SiR₃, wherein R is a linear or branched C1-C6 alkyl, cyclic C1-C6 alkyl, optionally substituted with -OH, -NH₂ or halogen; at least one of the remaining R₁-R₅ are selected from one or more group(s) selected from the group consisting of a substituted amine with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a -(O-CH₂-CH₂)₁₋₅-OCH₂-COOH, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, or an amine; and any R₁-R₅ remaining thereafter is/are H;

and wherein R₆ is a H.

8. A compound as defined in any of claims 1-6 wherein the radionuclide is ²¹¹At or ¹³¹I for use in radionuclide therapy.
9. A compound for use as defined in claim 8, wherein the radionuclide therapy is of a cancer disease.
10. A compound for use as defined in claim 8, wherein the radionuclide therapy is applied to target vectors which do not internalize, such as pathogen targets and/or cells infected with pathogens.
11. A compound for use as defined in any of claims 8 to 10, wherein the compound does not penetrate cell membranes.

12. A method comprising reacting



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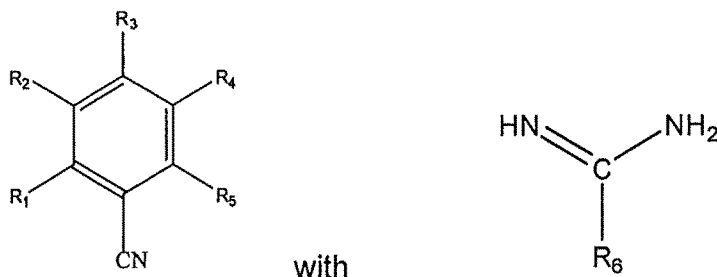
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25

30

wherein one of R_1 - R_5 is independently selected from the group consisting of: SnR_3 , B(OR)_2 , $\text{I}^+\text{-Ar}$, I double-bonded to R or SiR_3 , wherein R is a linear or branched C1-C6 alkyl, cyclic C1-C6 alkyl, optionally substituted with $-\text{OH}$, $-\text{NH}_2$ or halogen; and at least one of the remaining R_1 - R_5 are selected from the group consisting of a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(\text{O}-\text{CH}_2-\text{CH}_2)_{1-5}-\text{OCH}_2-\text{COOH}$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, an substituted amine; with 1-5 polyethylene glycol unit(s), a $-(\text{O}-\text{CH}_2-\text{CH}_2)_{1-5}-\text{OCH}_2-\text{COOH}$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, or an amine; and any R_1 - R_5 remaining thereafter is/are H; and wherein R_6 is a H.

13. A method according to claim 12 comprising reacting



with

wherein one of R₁-R₅ are independently selected from I or F and at least one of the remaining R₁-R₅ are selected from the group consisting of a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, (C1-C10) alkyl, (C2-C10)alkenyl, (C2-C10)alkynyl, (C1-C10)alkylene, (C1-C10)alkoxy, (C2-C10)dialkylamino, (C1-C10)alkylthio, (C2-C10)heteroalkyl, (C2-C10)heteroalkylene, (C3-C10)cycloalkyl, (C3-C10)heterocycloalkyl, (C3-C10)cycloalkylene, (C3-C10)heterocycloalkylene, (C1-C10)haloalkyl, (C1-C10)perhaloalkyl, (C2-C10)-alkenyloxy, (C3-C10)-alkynyloxy, aryloxy, arylalkyloxy, heteroaryloxy, heteroarylalkyloxy, (C1-C6)alkyloxy-(C1-C4)alkyl, optionally substituted aryl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted means one or more substituents selected from a halogen, an hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, amine, a substituted amine with 1-5 polyethylene glycol unit(s), a $-(O-CH_2-CH_2)_{1-5}-OCH_2-COOH$, H, Methyl, Ethyl, Propyl, optionally substituted heteroaryl, and optionally substituted arylalkyl; wherein optionally substituted in relation to said substituted amine means one or more substituents selected from a halogen, a hydroxy group, a sulfonamide, a carboxyl group, a sulfonyl group, or an amine; and any R₁-R₅ remaining thereafter is/are H;

and wherein R₆ is a H.

14. A method according to claim 12 or 13, wherein the reaction is carried out at a temperature range of from 50 to 70°C and wherein water is added after cooling to room temperature followed by addition of HCl and extraction with EtOAc.

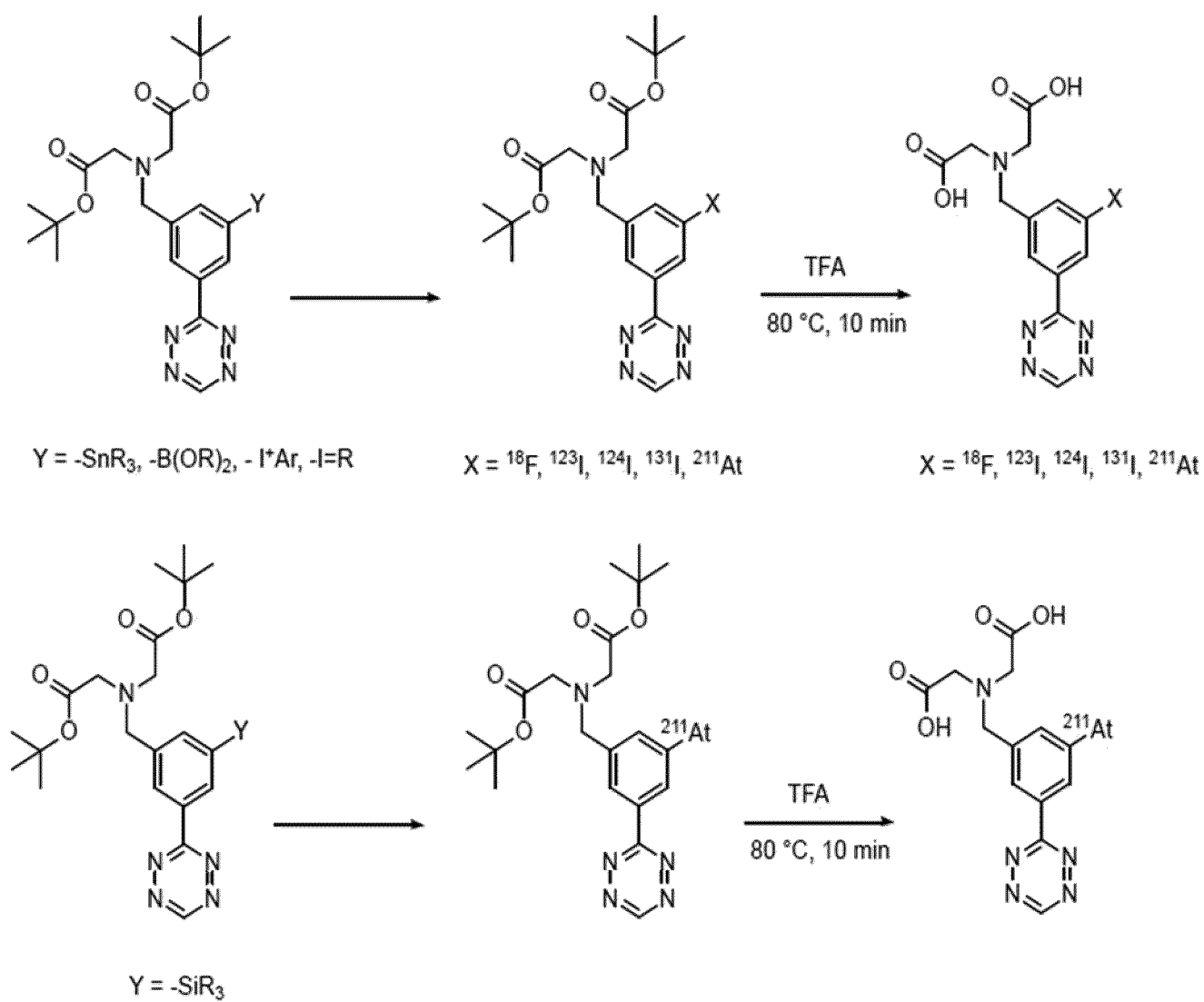
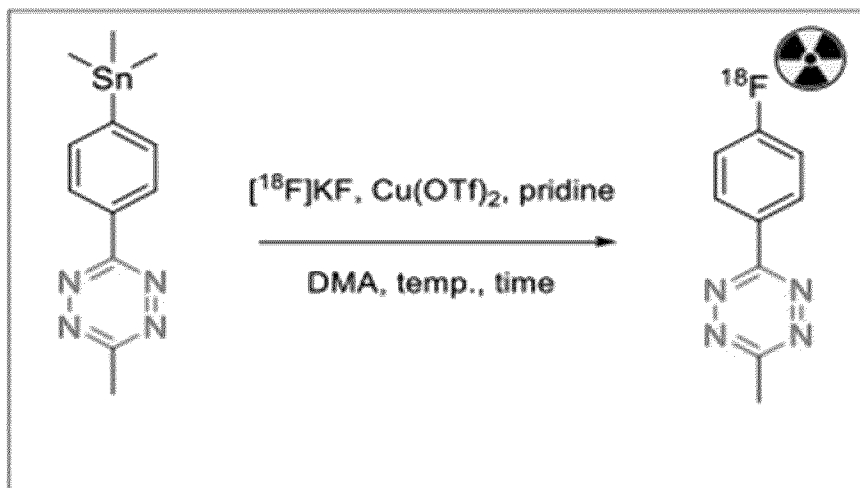


FIG. 1

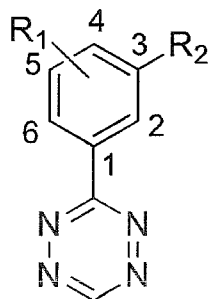


Base amount (μg) ^[a]	RCC (%)
25	29 $\pm$ 8
50	30 $\pm$ 5
100	19 $\pm$ 2
150	20 $\pm$ 5
200	9 $\pm$ 1

Temperature ($^{\circ}\text{C}$) ^[b]	RCC (%)
60	0
80	16 $\pm$ 8
100	30 $\pm$ 5
115	20 $\pm$ 3
130	14 $\pm$ 2

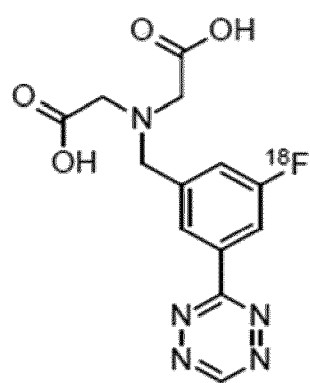
Reaction time (min) ^[c]	RCC (%)
1	0
3	29 $\pm$ 2
5	30 $\pm$ 5
10	15 $\pm$ 5
15	16 $\pm$ 2

FIG. 2

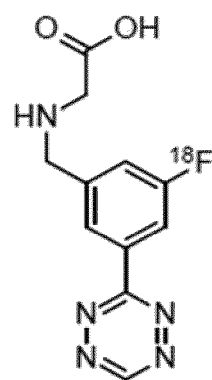


Tetra- zine	R ₁ Posi- tion	R ₁	R ₂	ClogD _{7.4} ^a	Rate constant (M ⁻¹ s ⁻¹) ^b	Normalized blocking effect
						10:1 Tz/Tco Ratio
RGV 52	-	-	-F	0.73	222 ^c	32,88
RGV 55	4	-F	-	0.75	159 ^c	4,85
UB-12	4	-COOH	-F	-3.2	80777	86,75
UB-19	4	-CH ₂ N(CH ₂ COOH) ₂	-H	-6.89	62004	96,72
UB-28	4	-CH ₂ NHCH ₂ COOH	-H	-3.03	60442	86,29
UB-37	4	-NHCO(CH ₂) ₃ COOH	-F	-2.93	72611	89,3
UB-44	4	-CH ₂ N(CH ₂ COOH) ₂	-F	-6.91	76234	97,93
UB-39	4	-CH ₂ NHCH ₂ COOH	-F	-2.98	76133	97,86
UB-40	4	-CH ₂ NHCO(CH ₂) ₃ COOH	-F	-3.34	74752	96,62
UB-50	4	-CH ₂ N(CH ₂ COOH) ₂	-I	-6.55	86461	92
UB-138	4	-CH ₂ NHCH ₂ COOH	-I	-2.20	87261	91,2
UB-80	5	-CH ₂ N(CH ₂ COOH) ₂	-H	-6.97	61743	94,49
UB-92	5	-CH ₂ NHCH ₂ COOH	-H	-3.03	55499	95,8
UB-108	5	-CH ₂ N(CH ₂ COOH) ₂	-F	-6.93	81697	91,89
UB-137	5	-CH ₂ NHCH ₂ COOH	-F	-2.89	77835	94,93

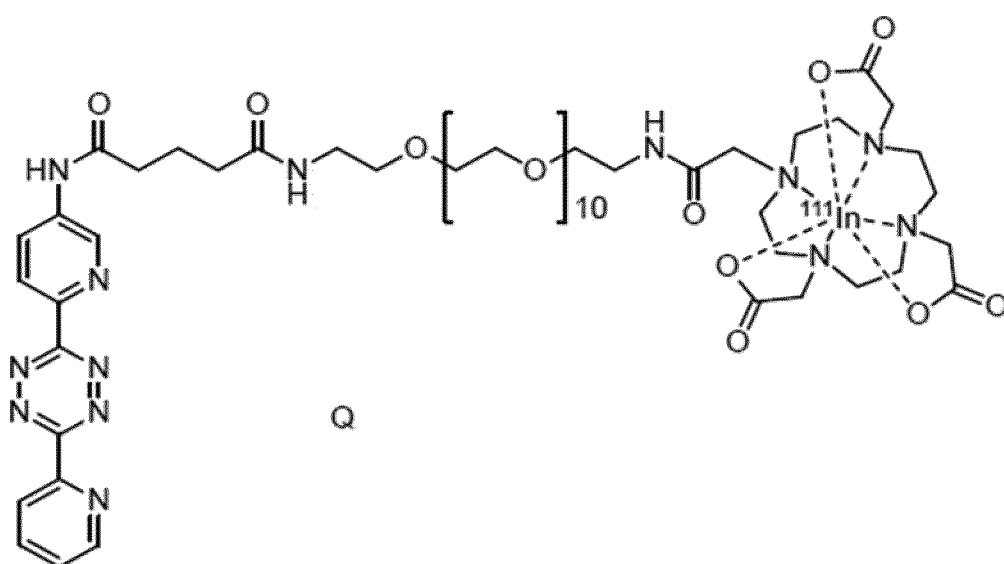
FIG. 3



UB_108



UB_137



Q

FIG. 4

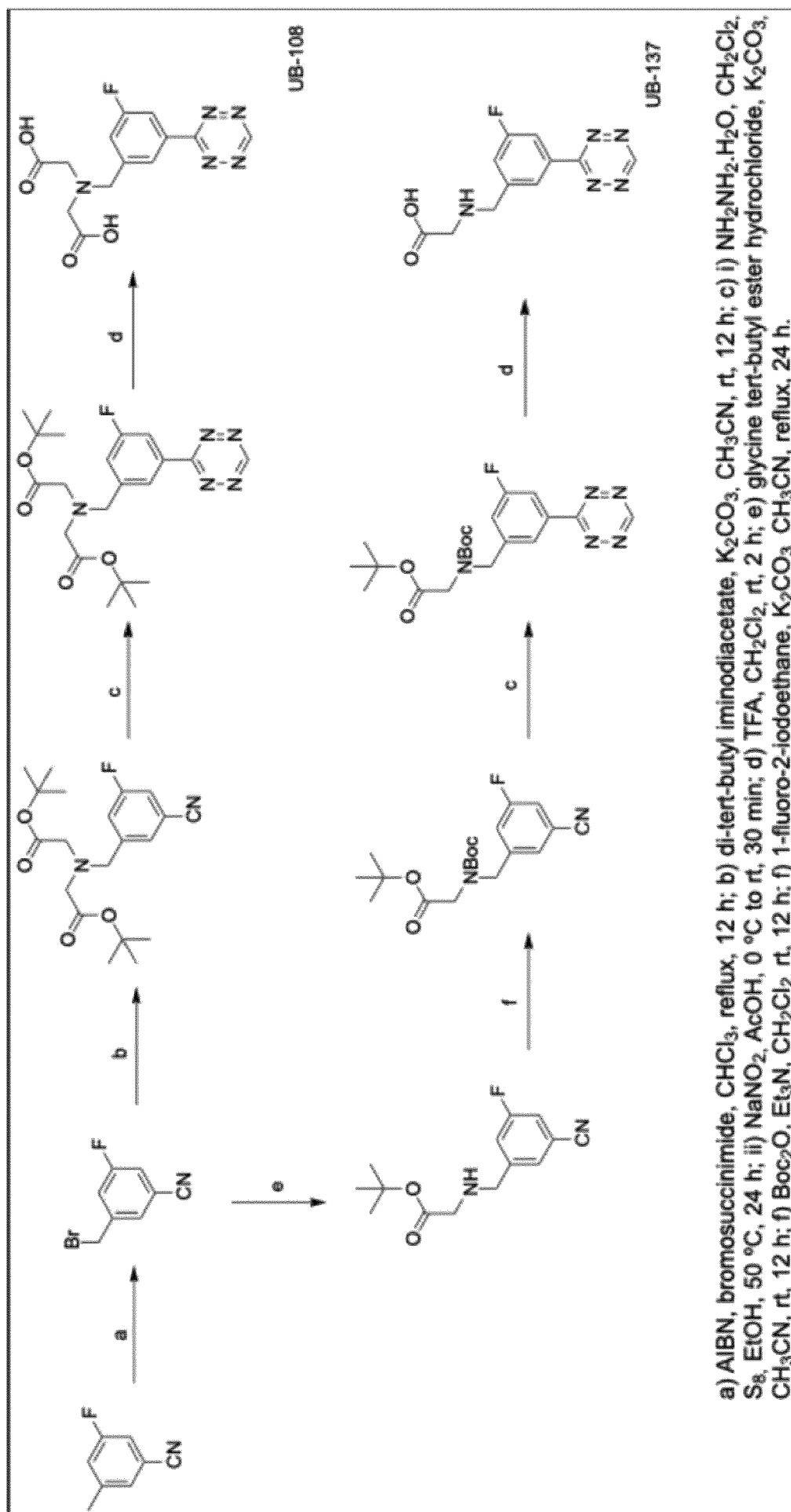
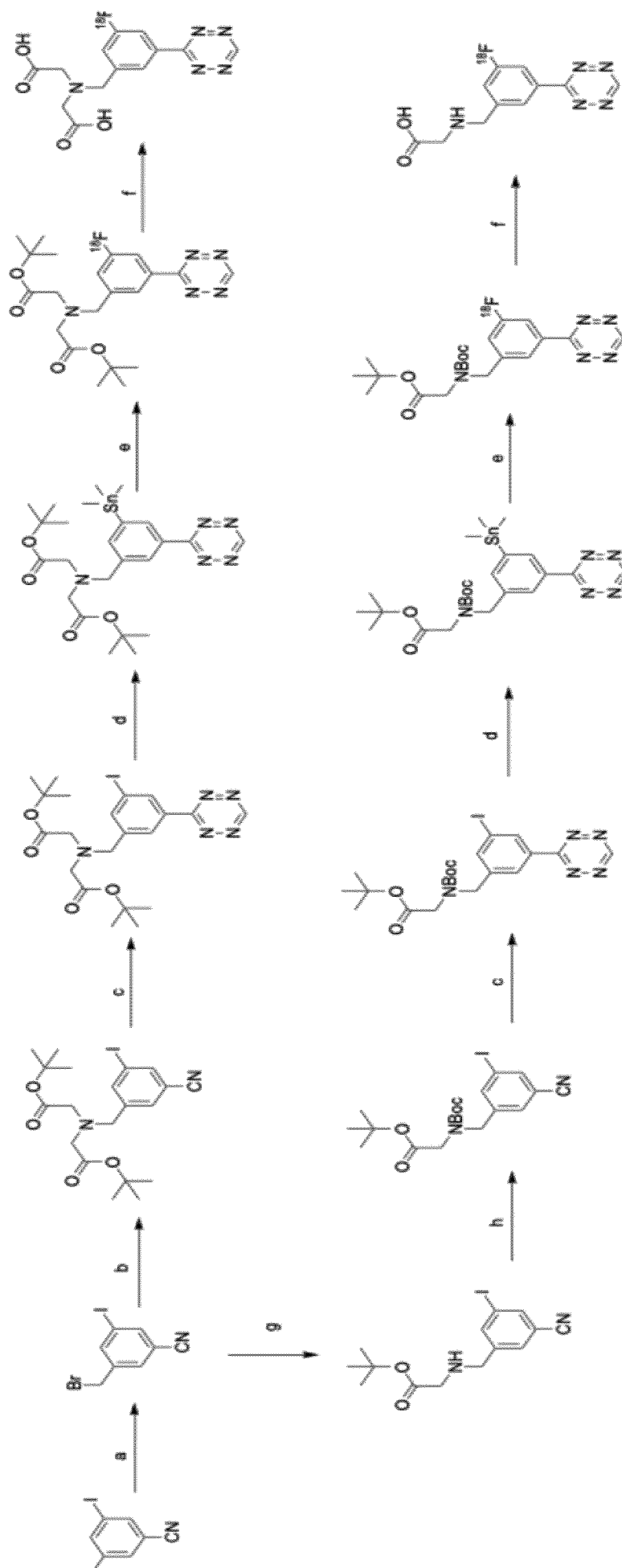


FIG. 5



a) AIBN, bromosuccinimide, CHCl_3 , reflux, 12 h; b) di-tert-butyl iminodiacetate, K_2CO_3 , CH_3CN , rt, 12 h; c) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, CH_2Cl_2 , S_8 , EtOH , 50°C , 24 h; d) NaNO_2 , AcOH , 0°C to rt, 30 min; e) Me_3SnI , $\text{Pd}(\text{PPh}_3)_4$, THF , 65°C , 2 h; f) $\text{Cu}(\text{OTf})_2$, pyridine, $[\text{F}]\text{KF}$, DMA, 100°C , 5 min; g) TFA, 80°C , 10 min; h) glycine tert-butyl ester hydrochloride, K_2CO_3 , CH_3CN , rt, 12 h; i) Boc_2O , Et_3N , CH_2Cl_2 , rt, 12 h.

FIG. 6

UB-108

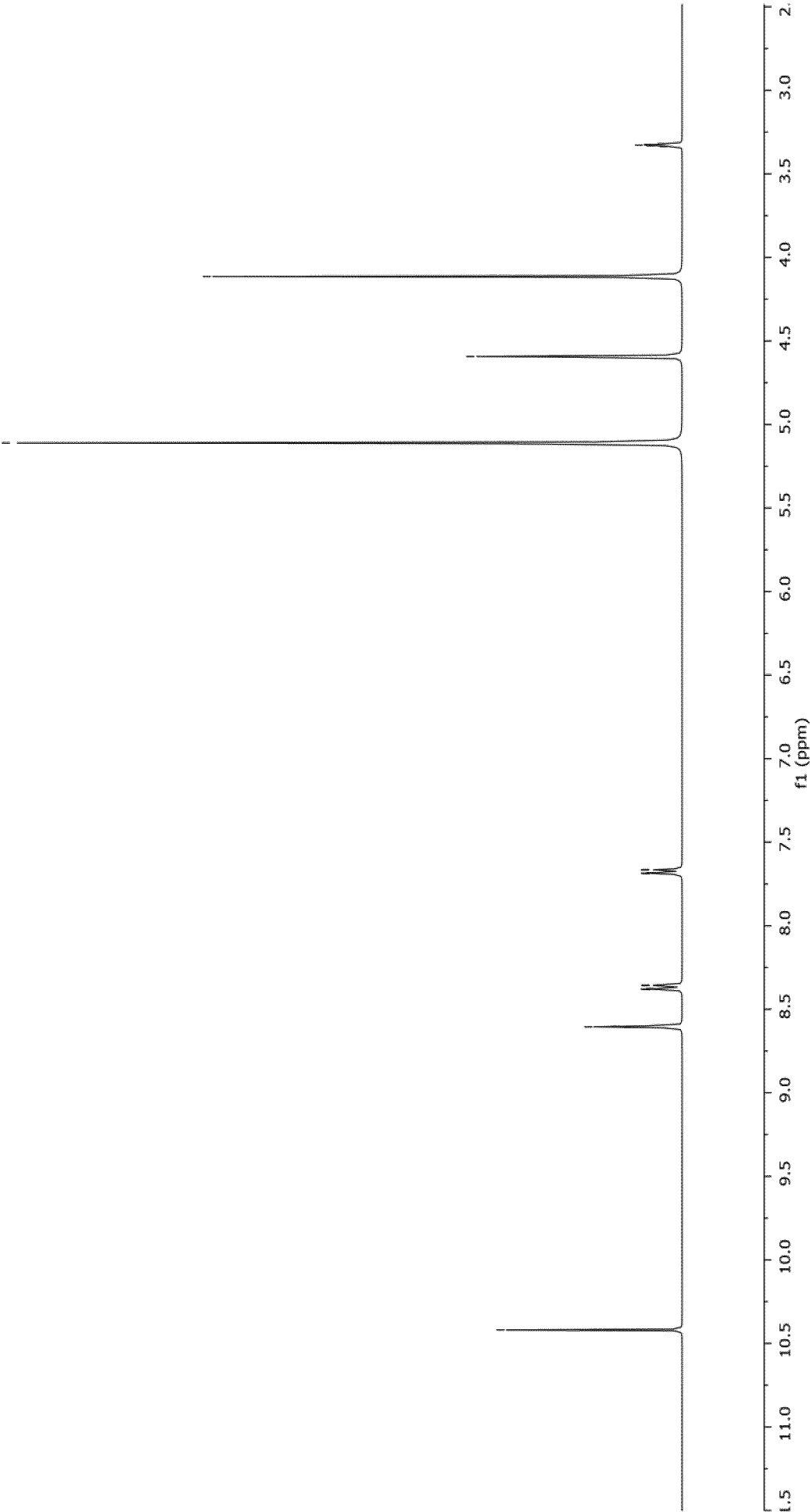


FIG. 7

UB-108 precursor, UB-161

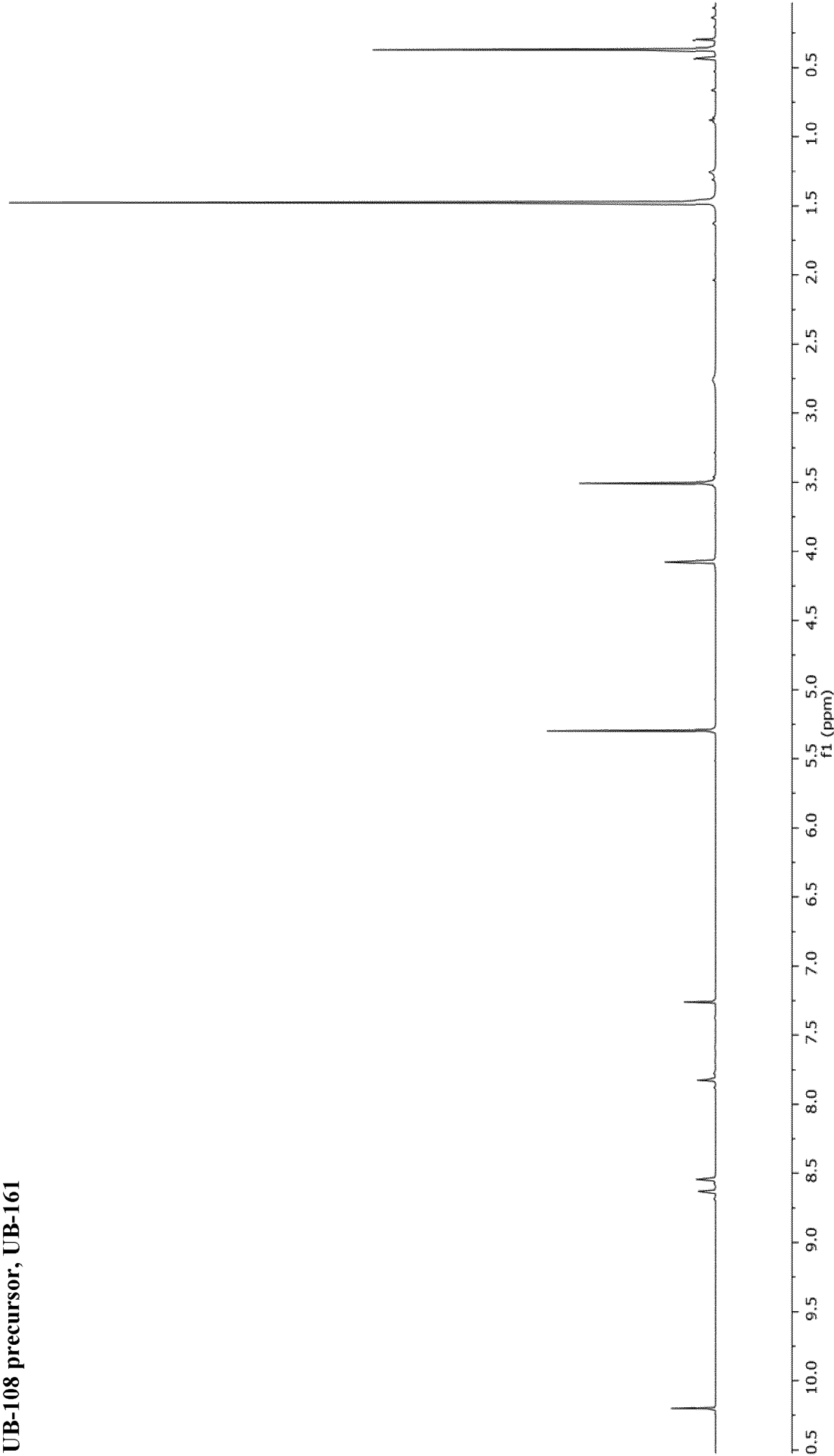


FIG. 7a

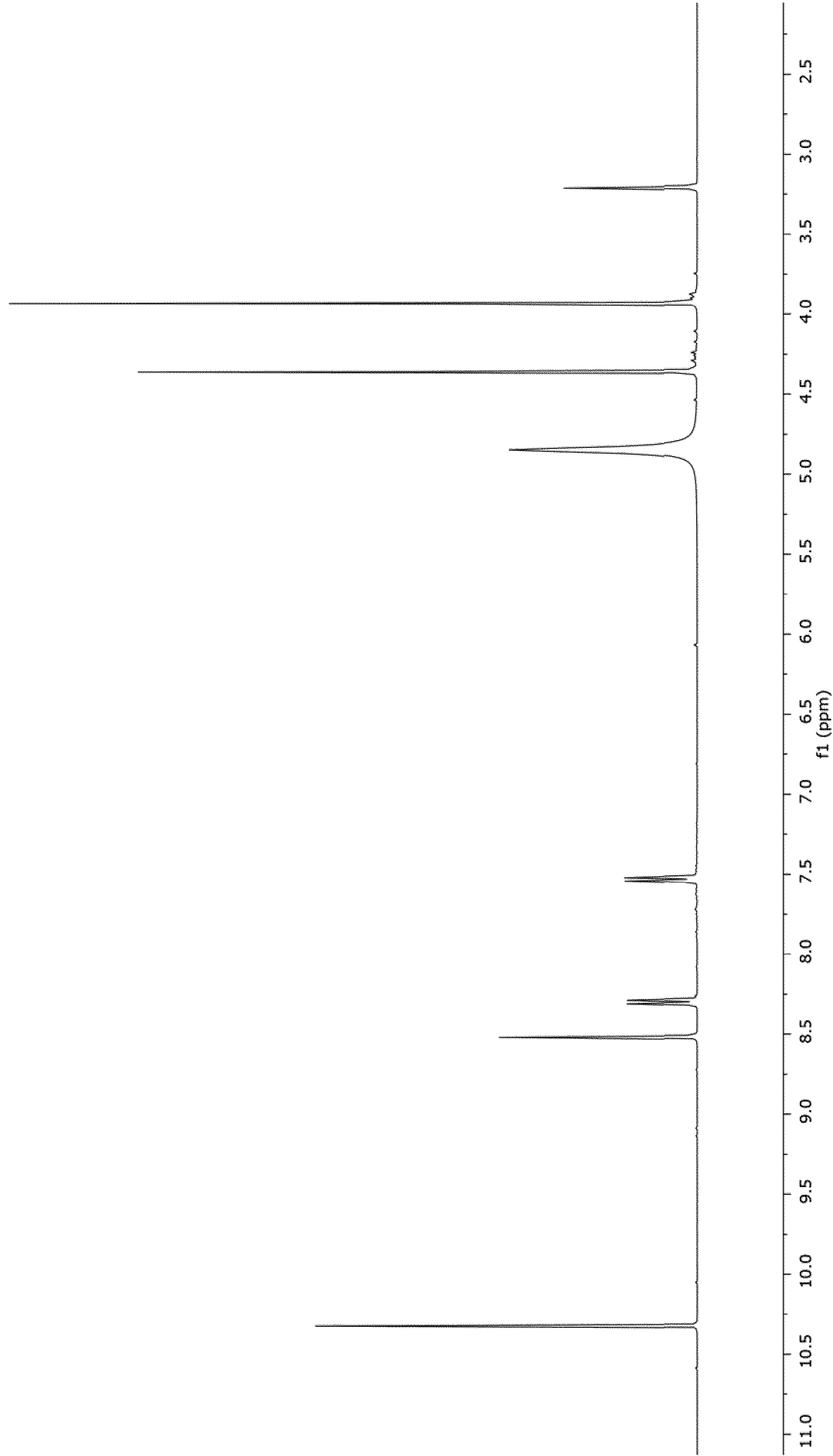


FIG 8

UB-137

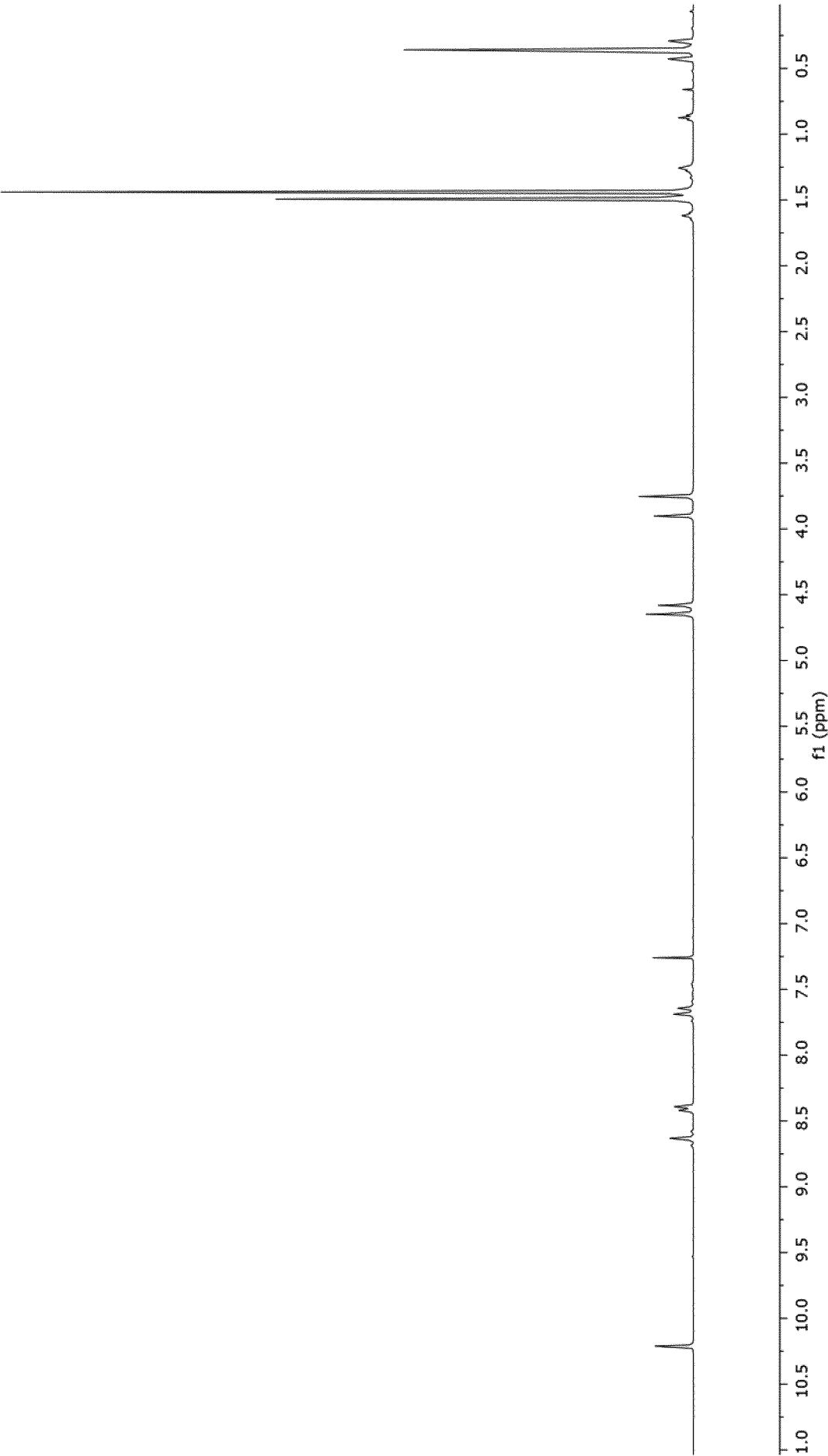


FIG. 8a

UB-137 precursor, UB-266

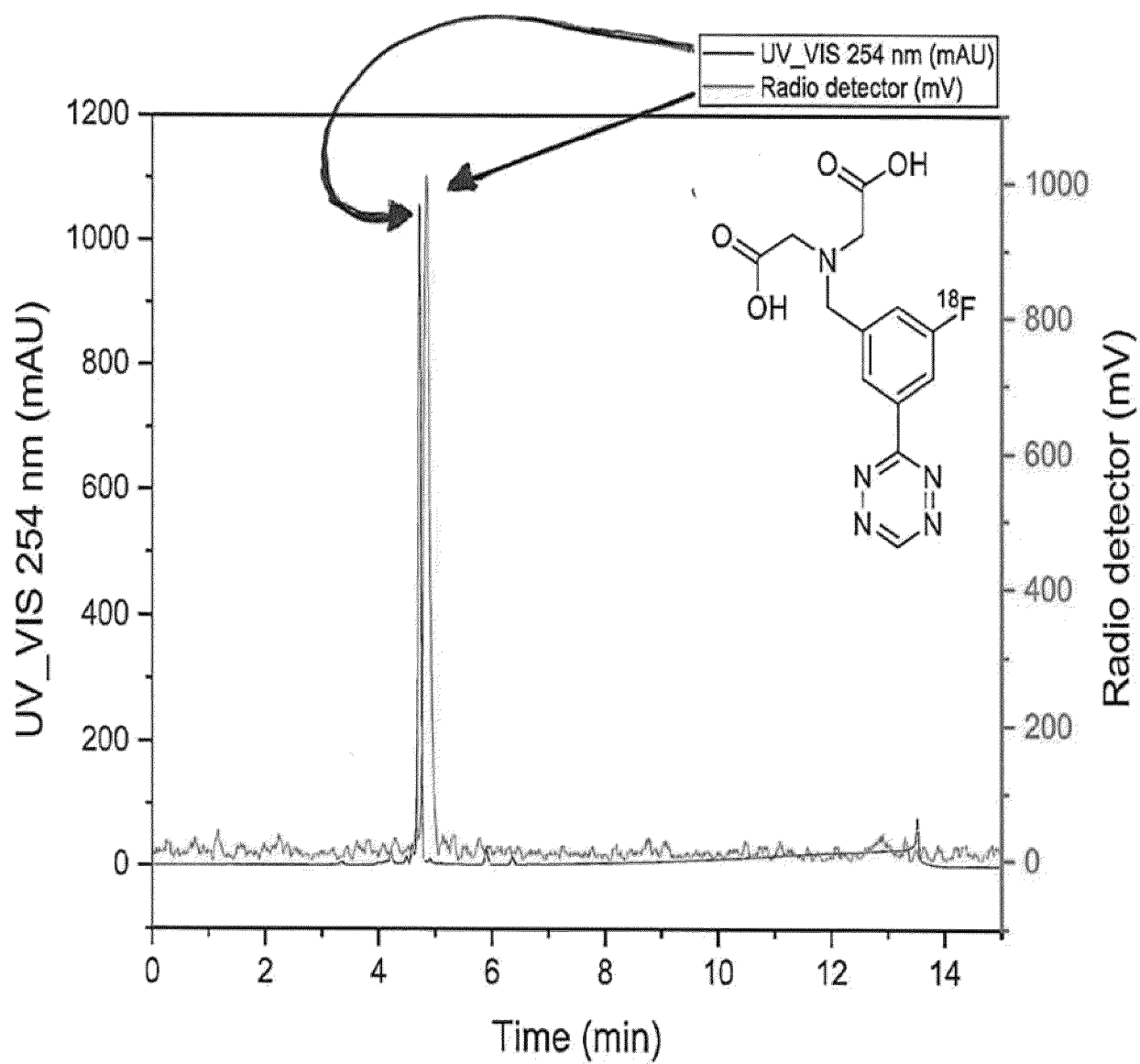


FIG. 9

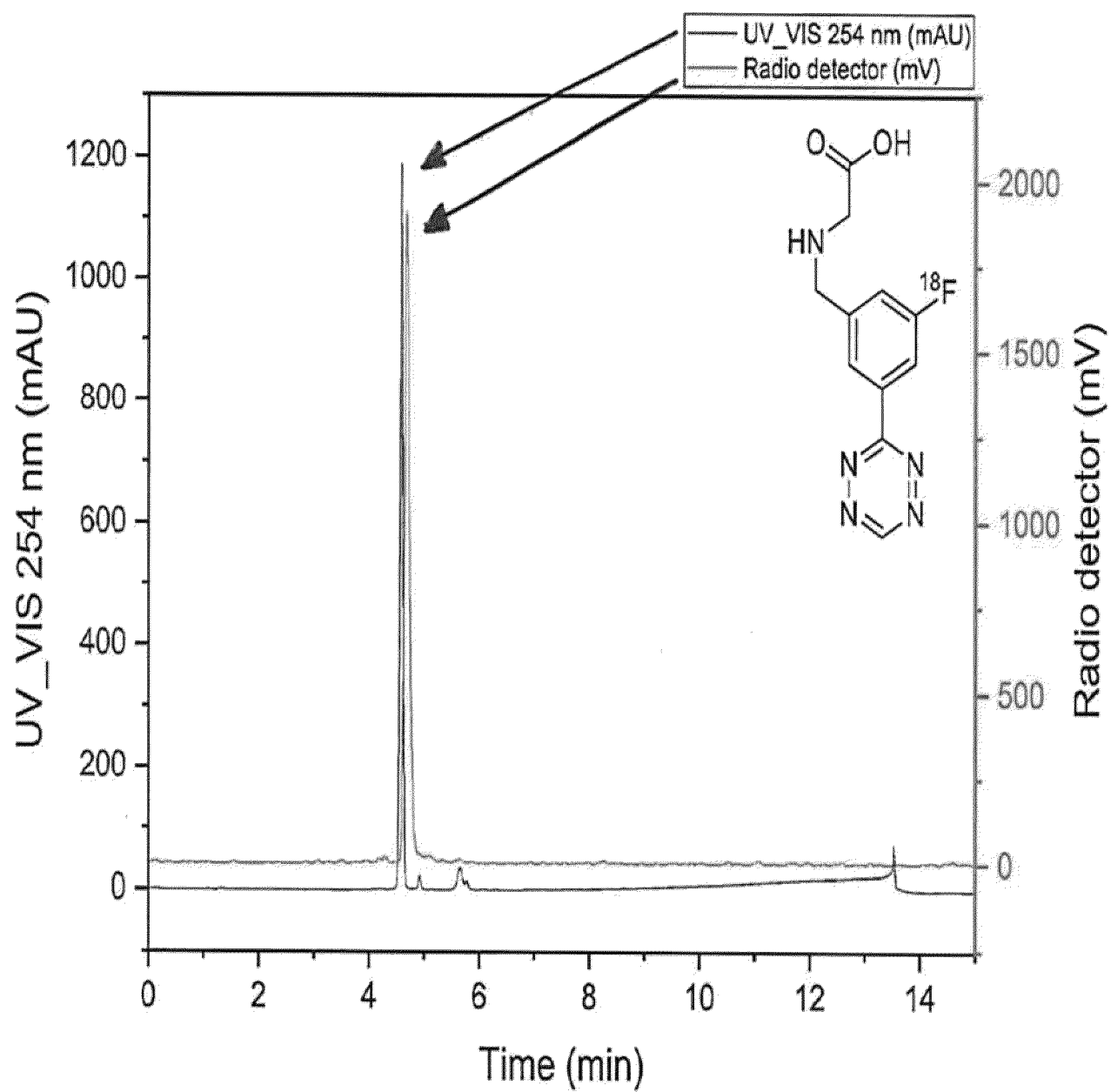


FIG. 10

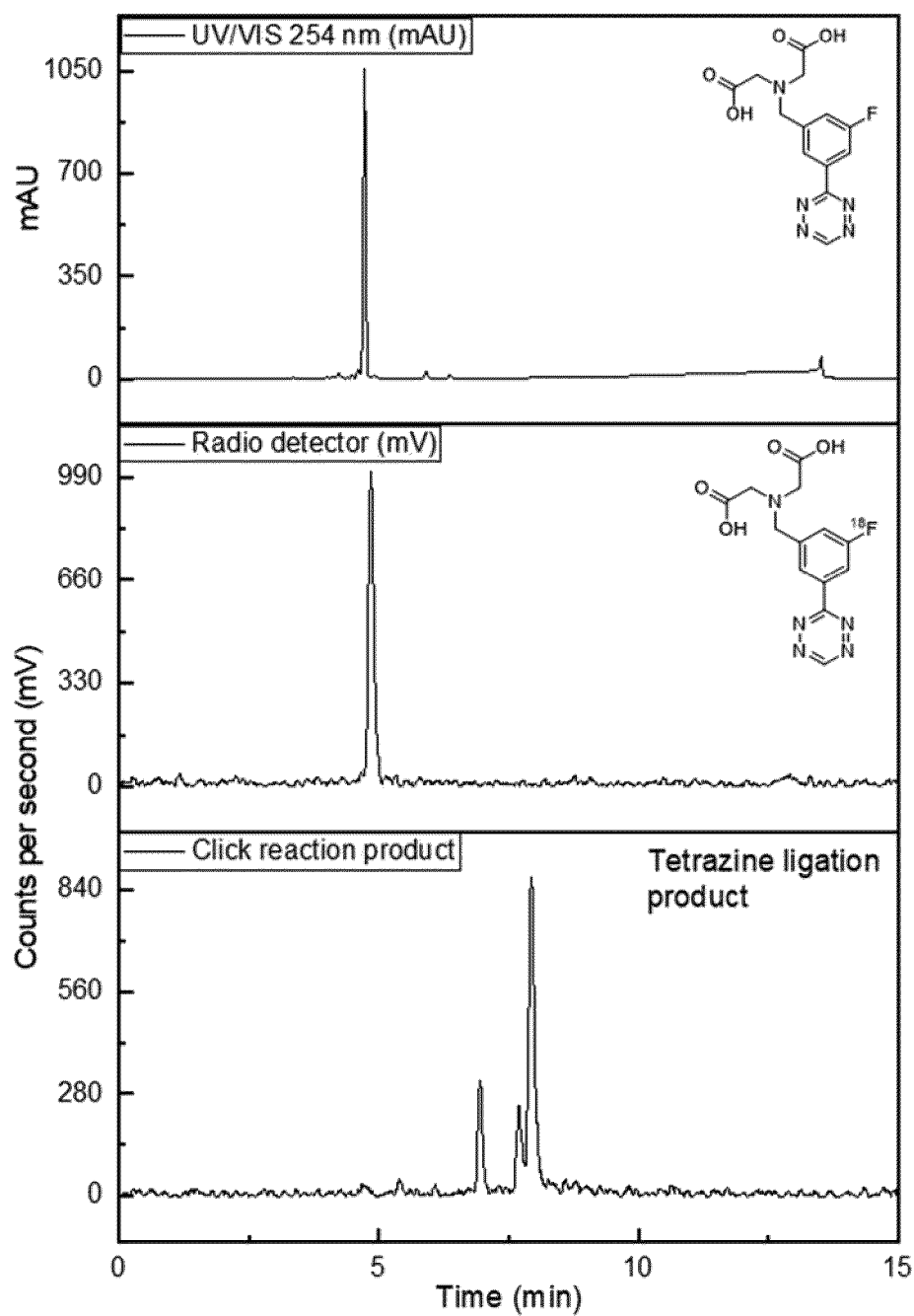
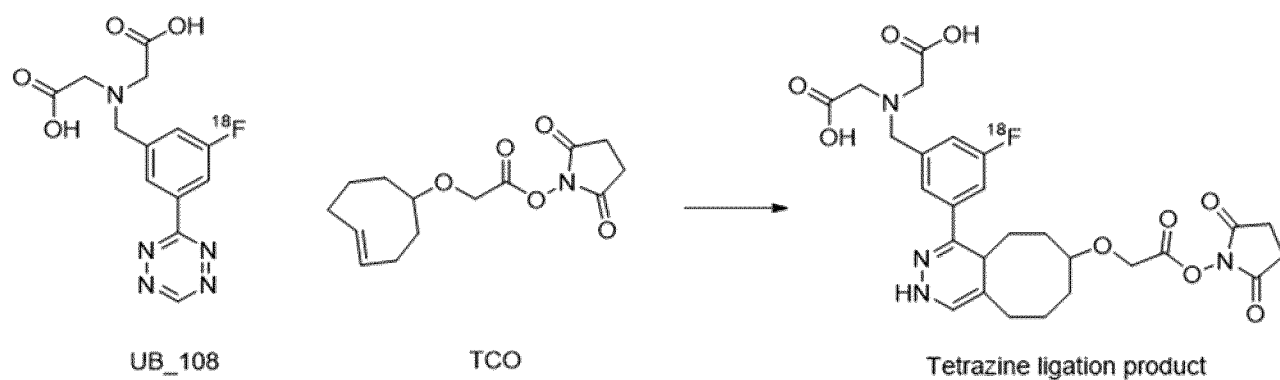


FIG. 11

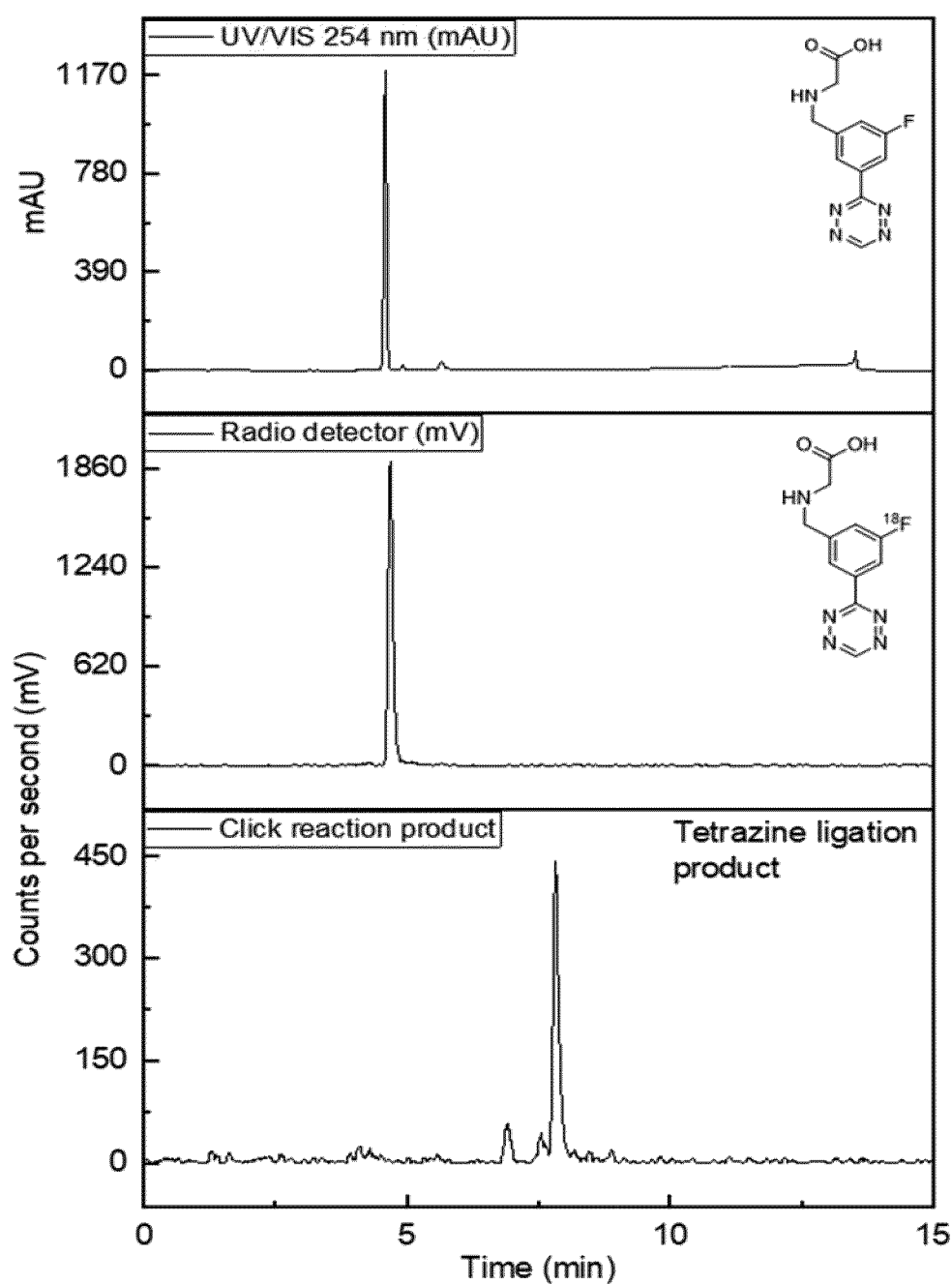
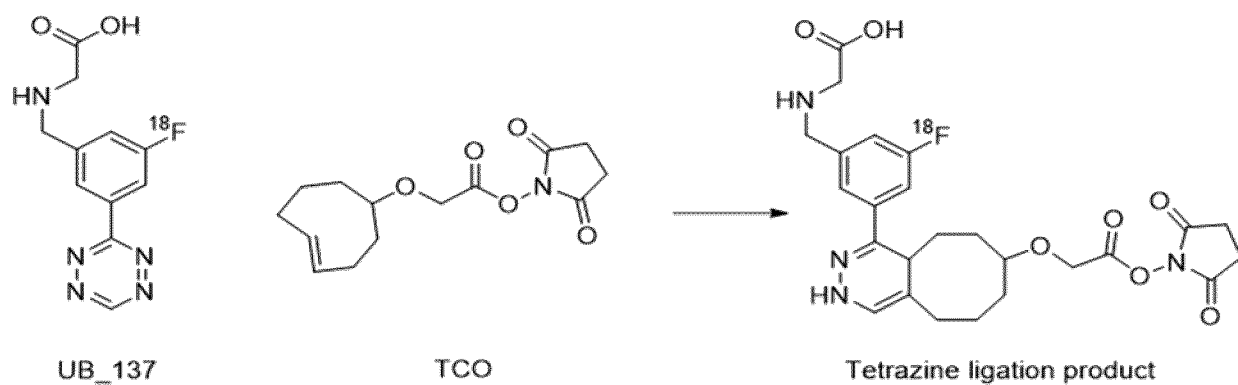


FIG. 11a



FIG. 12



FIG. 13

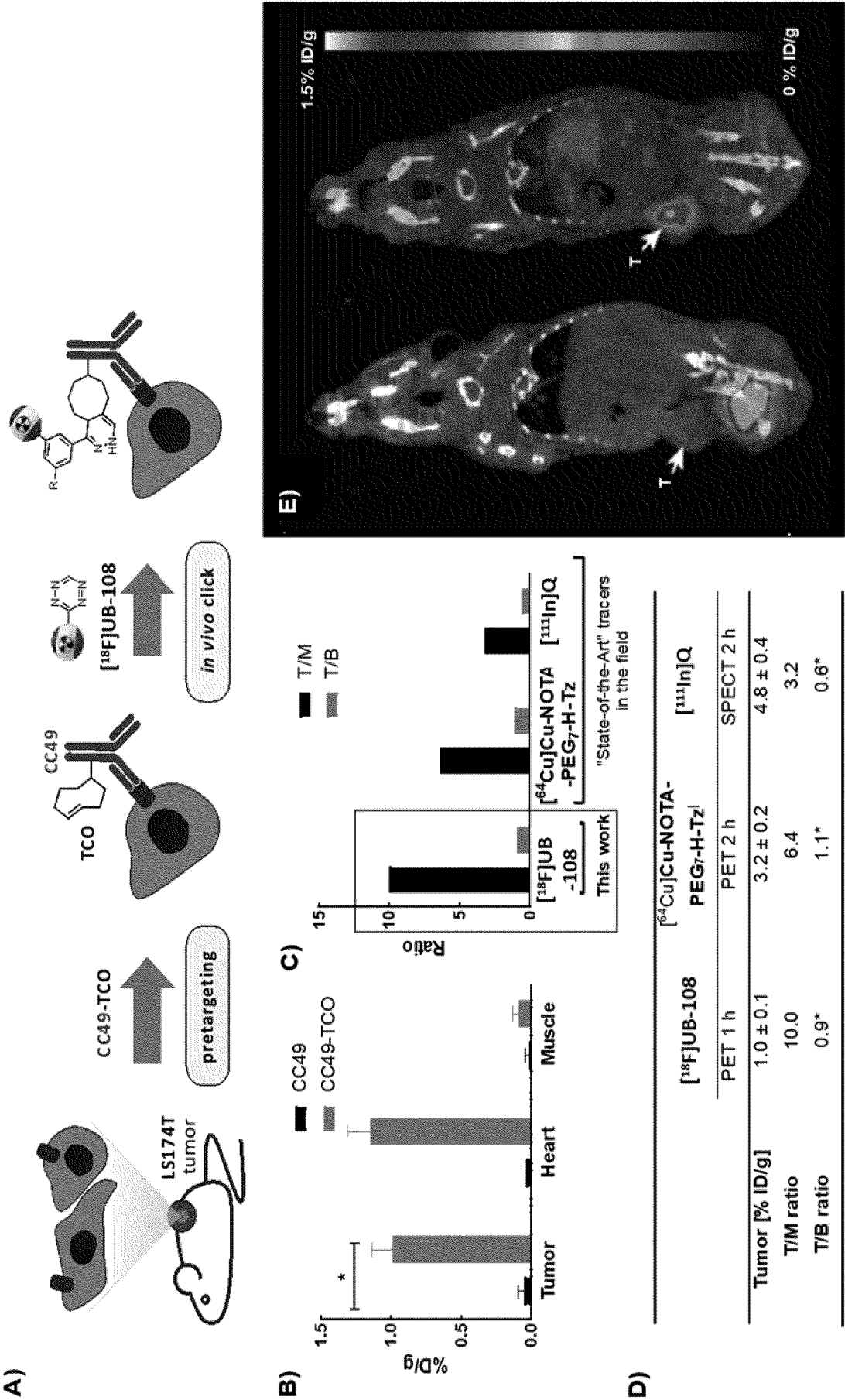


FIG. 14

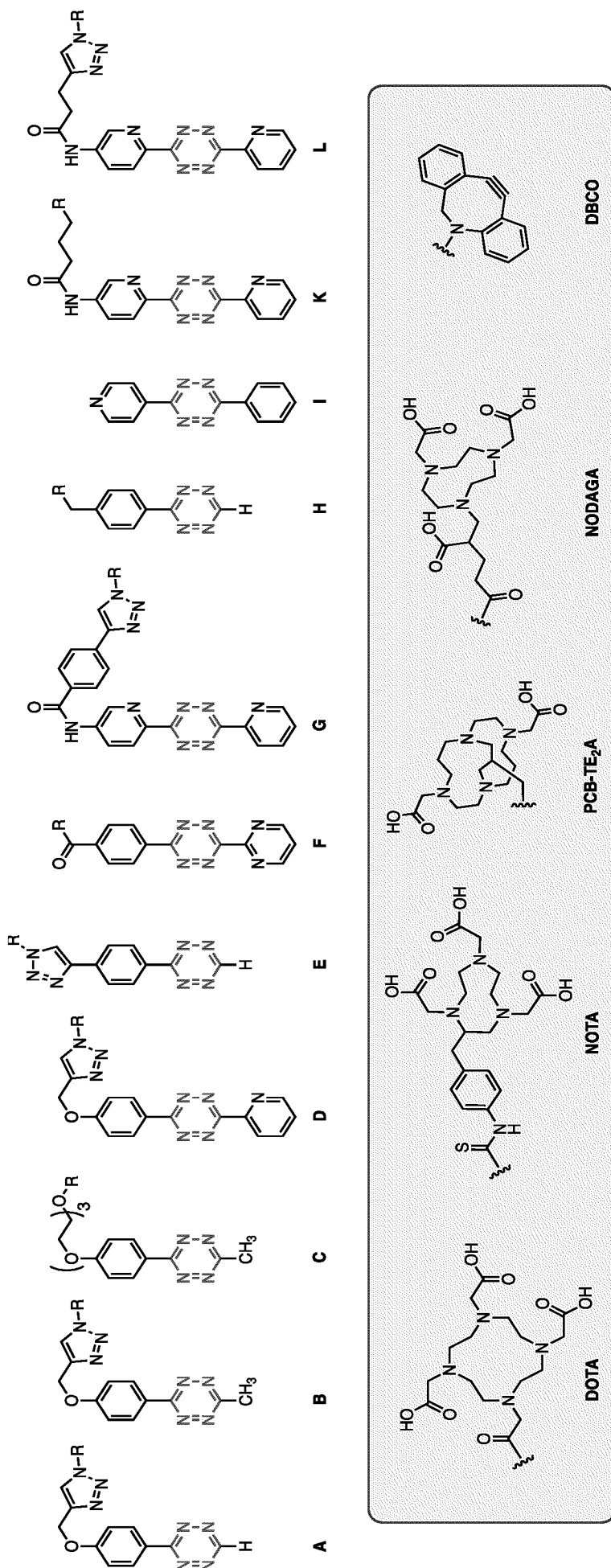


FIG. 15

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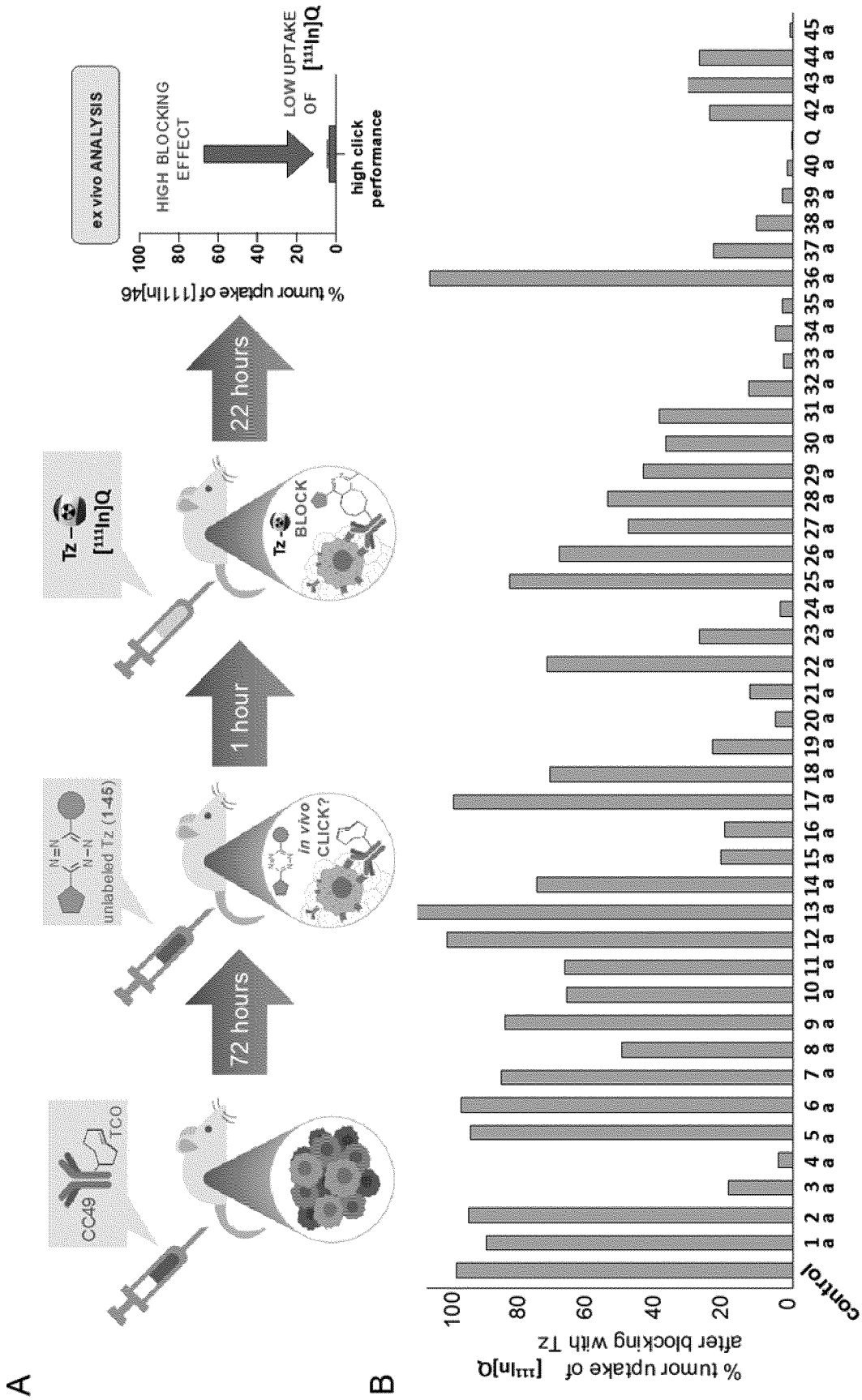


FIG. 16A + B

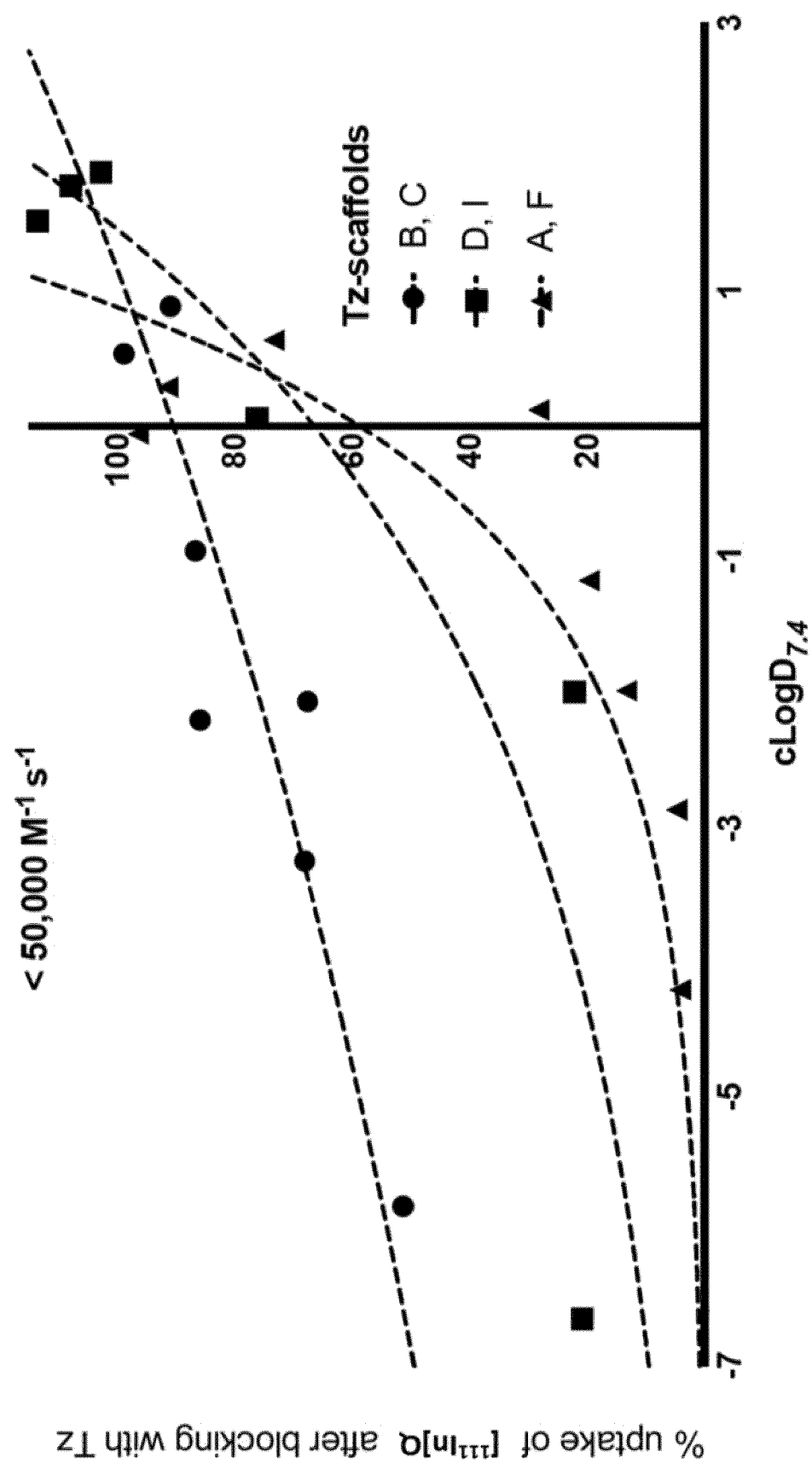


FIG. 16C

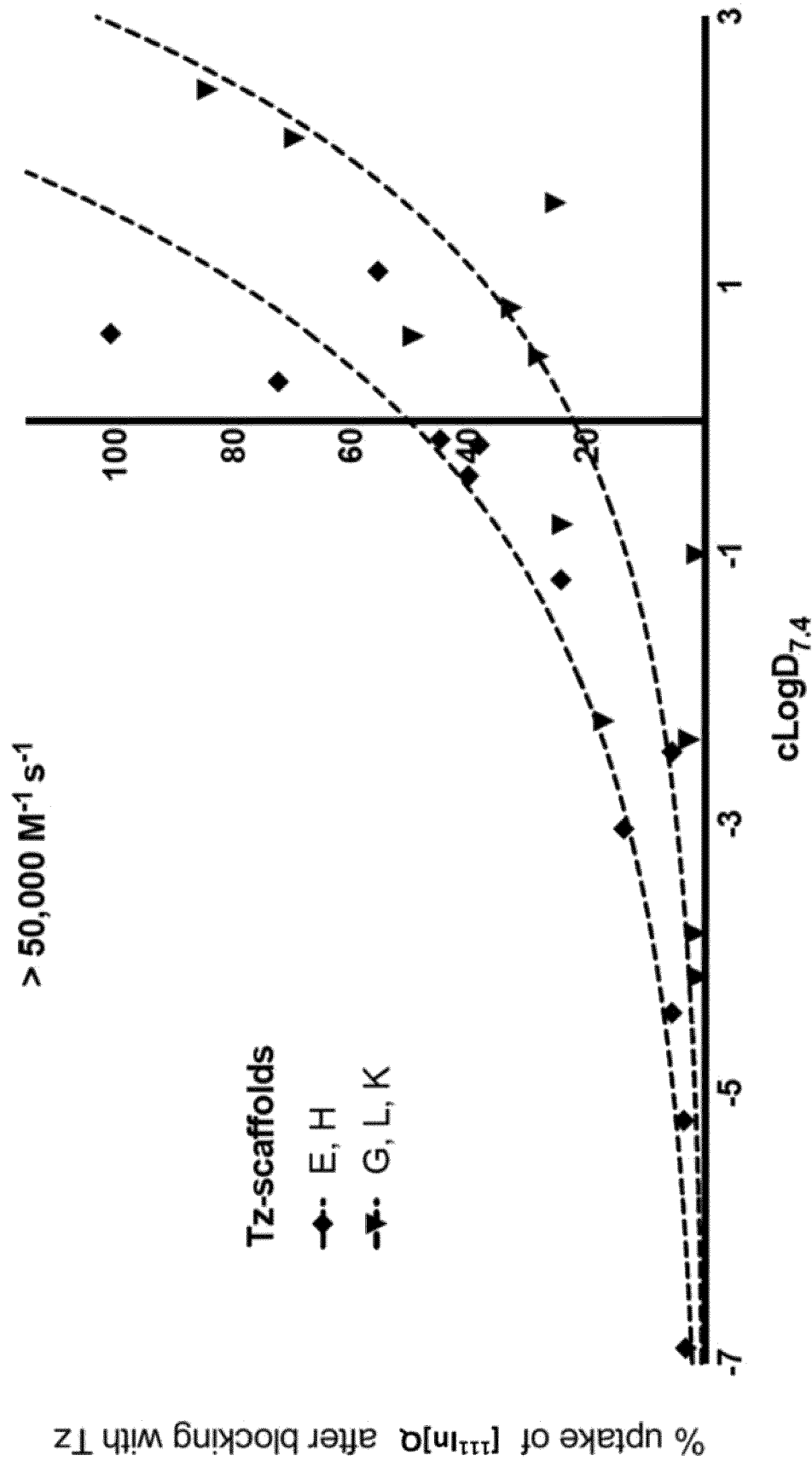


FIG. 16D

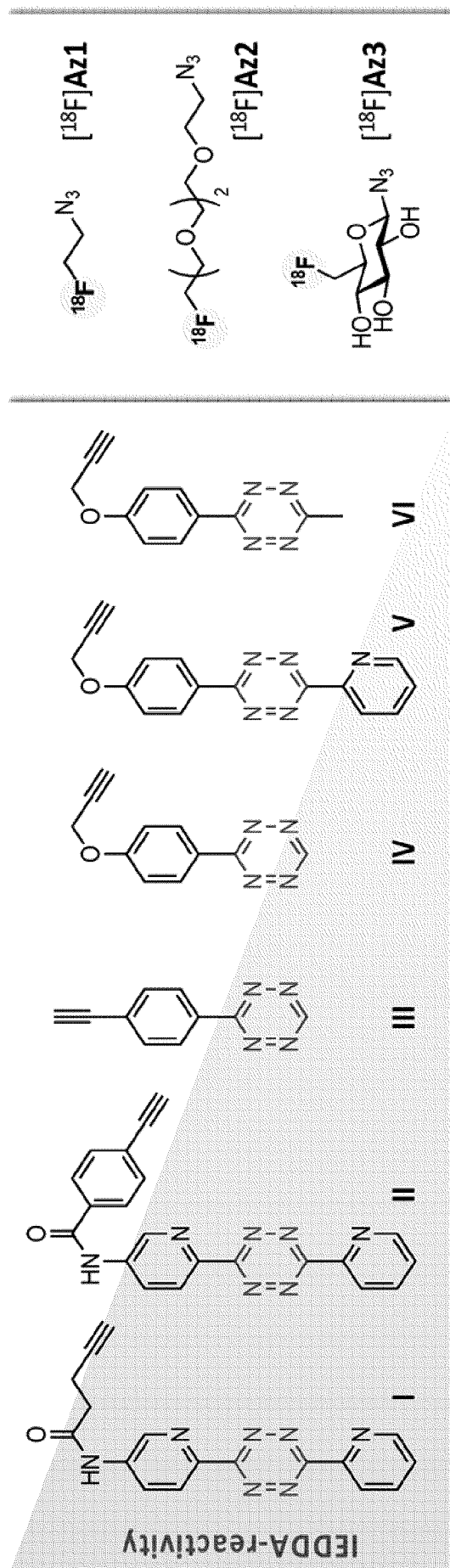
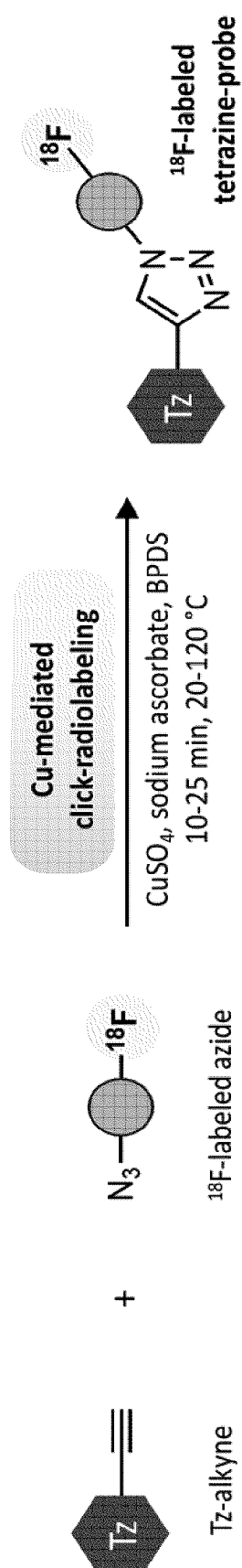


FIG. 17

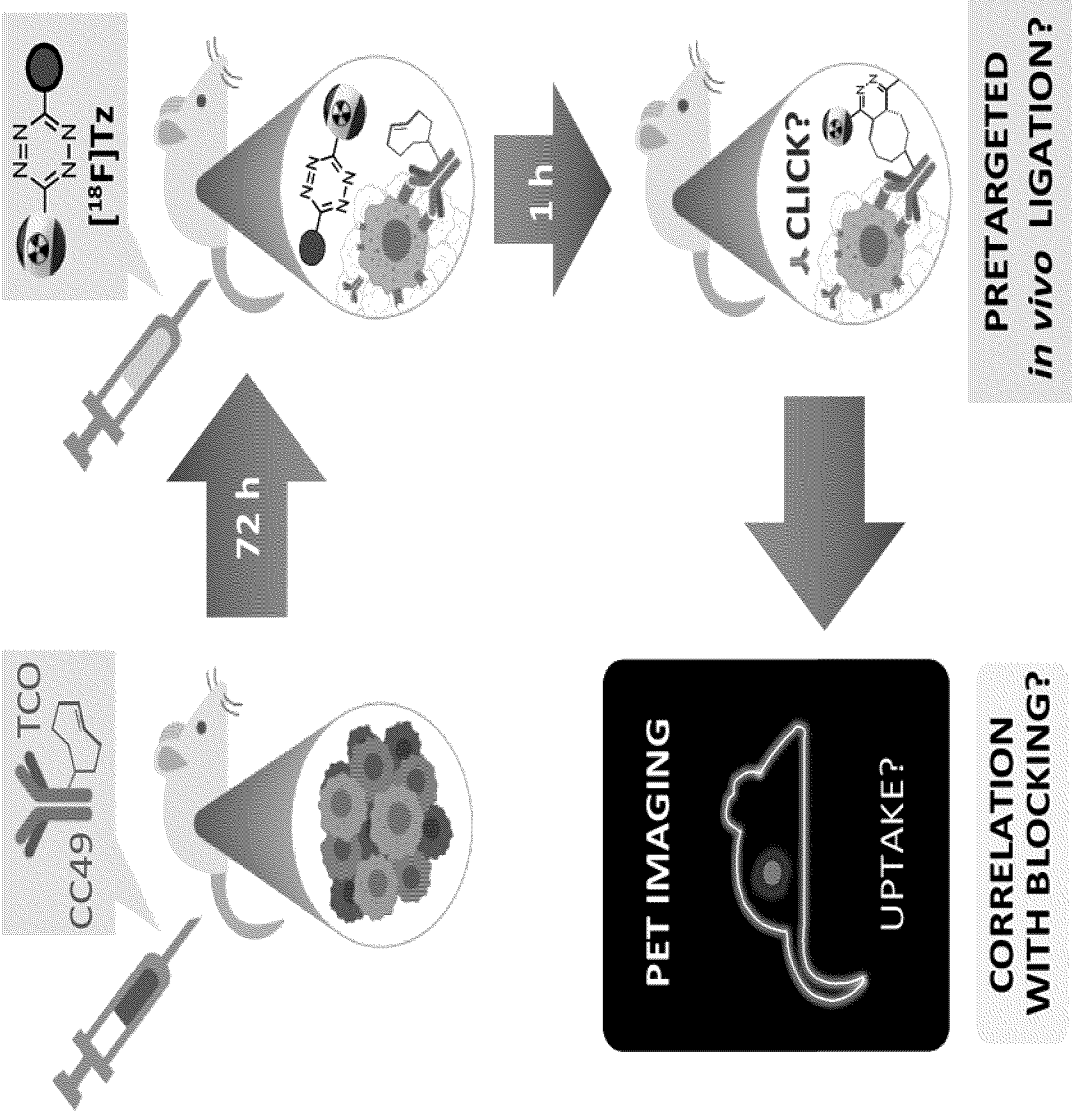


FIG. 18A

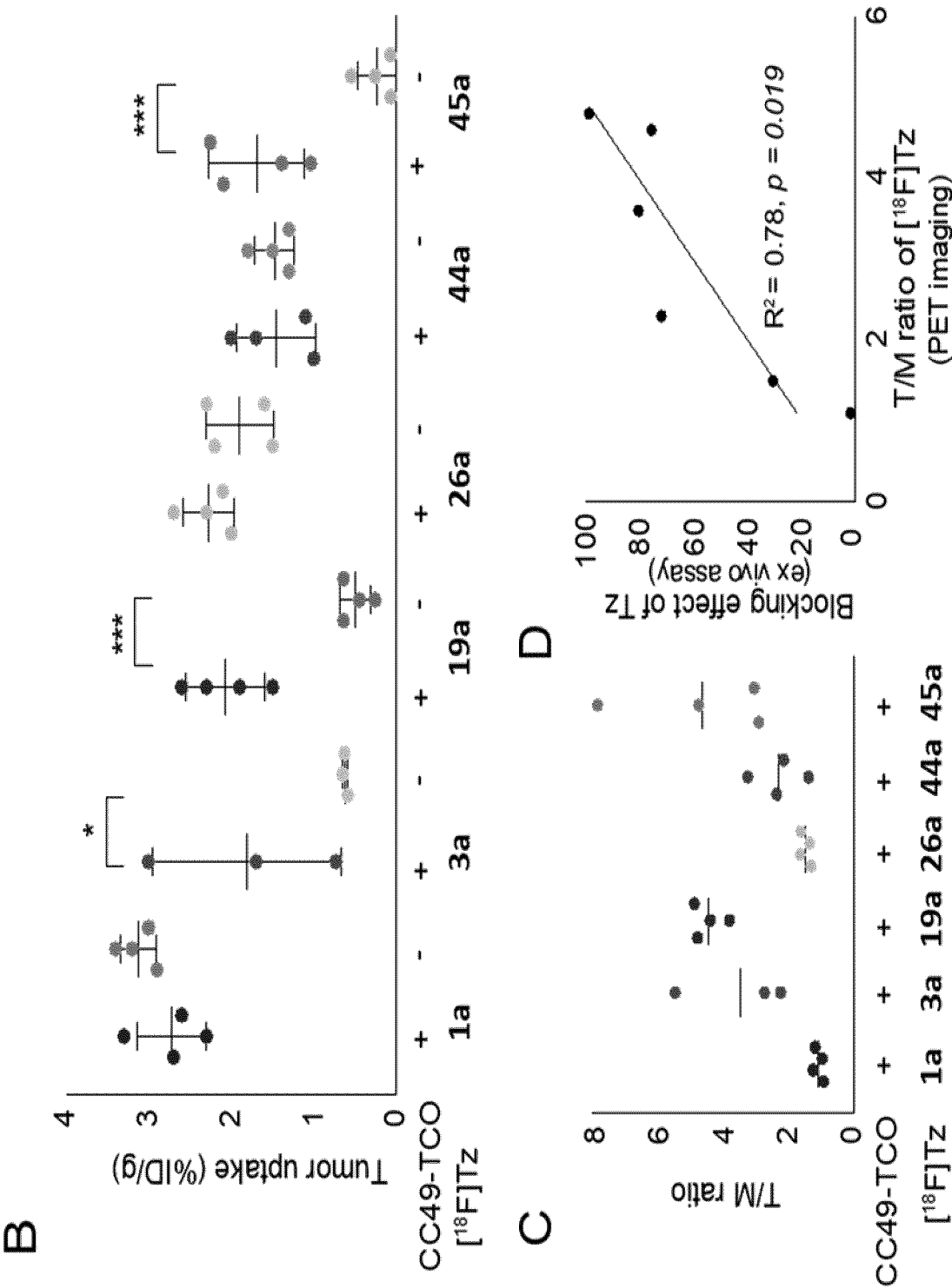


FIG. 18B + C + D

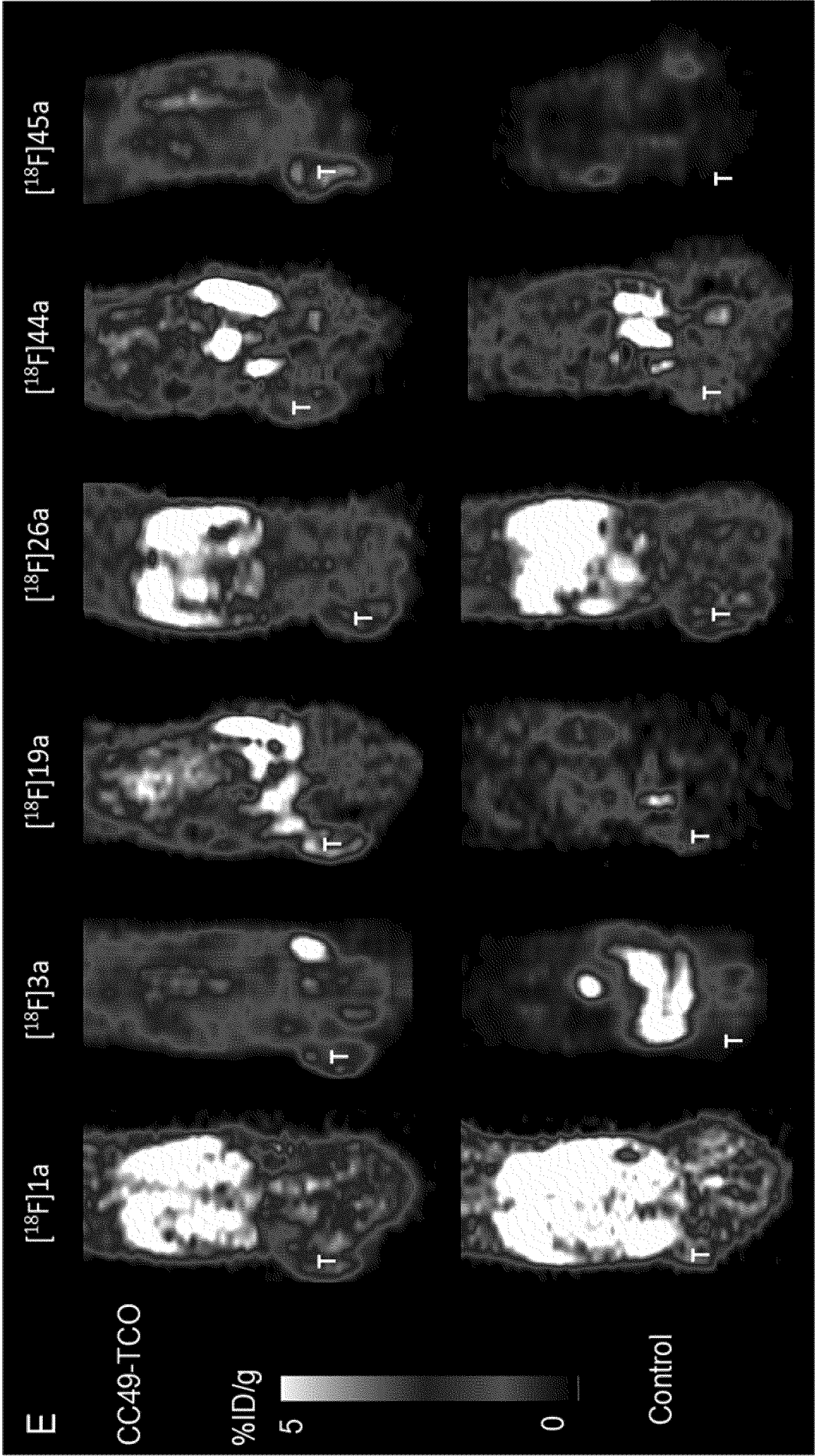


FIG. 18E

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/062712

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D257/08 C07F7/22 A61K51/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D C07F A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2020/108720 A1 (KOEbenhavns Univ [DK]; RIGSHOSPITALET [DK]) 4 June 2020 (2020-06-04) page 1, lines 8-11; page 3, lines 32-33; page 18, line 19 - page 19, line 3; -----	1-3,7, 12-14
A	WO 2014/065860 A1 (GEN HOSPITAL CORP [US]; DEVARAJ NEAL K [US]; KARVER MARK R [US]) 1 May 2014 (2014-05-01) the whole document -----	1-14
A	WO 2012/156918 A1 (KONINKL PHILIPS ELECTRONICS NV [NL]; ROBILLARD MARC STEFAN [NL] ET AL.) 22 November 2012 (2012-11-22) the whole document ----- -/-	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

5 July 2021

Date of mailing of the international search report

13/07/2021

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Kleidernigg, Oliver

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/062712

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARK R. KARVER ET AL: "Synthesis and Evaluation of a Series of 1,2,4,5-Tetrazines for Bioorthogonal Conjugation", BIOCONJUGATE CHEMISTRY, vol. 22, no. 11, 16 November 2011 (2011-11-16), pages 2263-2270, XP055122288, ISSN: 1043-1802, DOI: 10.1021/bc200295y Scheme 1 -----	1-14
A	WO 2014/081299 A1 (TAGWORKS PHARMACEUTICALS B V [NL]) 30 May 2014 (2014-05-30) page 113, preparation of compound (14) -----	12-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/062712

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